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Title: **Transferrin Binding Proteins of *Pasteurella haemolytica* and Vaccines Containing Same**

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Title: Transferrin B[redacted]urella haemolytica and Vaccines Containing Same 11/29/96

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FIELD OF THE INVENTION

The invention relates to novel transferrin binding proteins of *Pasteurella haemolytica*, truncations, analogs, homologs and isoforms thereof; nucleic acid molecules encoding the proteins and truncations, analogs, and homologs of the proteins; vaccines containing the proteins; antibodies against the proteins; and, uses of the proteins and nucleic acid molecules.

BACKGROUND OF THE INVENTION

Members of the genus *Pasteurella* comprise a group of related bacterial species that are important pathogens of ruminants. This group includes the species *Pasteurella haemolytica* which has been classified into two biotypes, A and T, on the basis of sugar utilization, and into 16 serotypes which are recognized on the basis of their somatic antigens (Biberstein, E. L. et al., 1960; Fraser et al., 1982). The T-type strains of *P. haemolytica*, characterized by utilization of trehalose, have been recently reclassified as a new species *P. trehalosi* (Sneath, P.H.A. et al., 1990).

Pneumonic pasteurellosis caused by *Pasteurella haemolytica* is a major economic problem to the cattle, sheep and goat industries world-wide. Shipping fever, a variation of this disease, is a major problem in the cattle industry in North America and is almost exclusively caused by type A1 strains of this species (Babiuk, L.A. and S. D. Acres, 1984). Serotype A2 is the most prevalent disease-causing type in sheep but other serotypes may be important in sheep and goats (Gilmour and Gilmour, 1991). The related species, *Pasteurella trehalosi* (formerly know as T-type *P. haemolytica*) is the causitive agent of septicemia in lambs, a problem plaguing the sheep industry particularly in the United Kingdom. Similarly, strains of the related species *Pasteurella multocida*, are responsible for haemorrhagic septicemia, a serious infection in cattle and water buffalo, which is particularly serious in South East Asia.

Vaccination is a desired method of control for pasteurellosis in ruminants but success has been limited by the lack of immunizing preparations that induce protection against all disease-causing serotypes, particularly if a vaccine effective for all ruminants is considered. Killed whole cell vaccines elicited inconsistent levels of protection and antibody response in calves (Wilkie, B.N., 1980). Homologous vaccines containing sodium salicylate extracts (SSEs) protected sheep against diseases due to serotypes A1, A6 and A9 (Gilmour et al., 1983) but not against the more epidemic serotype A2 (Fraser et al., 1982). An exotoxin produced by *P. haemolytica* which is specifically lethal to leucocytes and alveolar macrophages from ruminants (Benson et al., 1978) has shown a lot of promise as a vaccine candidate in protection experiments in calves and sheep (13,35) but there is limited protection against heterologous

serotypes (33). The inclusion of proteins induced under iron-limited growth conditions into a vaccine for pasteurellosis in lambs has been implicated in enhanced protection (15).

Previous studies have established that the ability of pathogenic bacteria to acquire iron *in vivo* is a critical factor in their pathobiology (7,11). One mechanism of iron retrieval from the host iron-binding glycoprotein, transferrin, involves direct binding of transferrin by surface receptors on the bacteria and the removal of iron from transferrin and uptake into the cell (21). Schryvers (1992) describes the isolation of transferrin receptor proteins from various bacterial pathogens using affinity chromatography. The transferrin receptor has been shown to consist of two proteins, called transferrin binding protein 1 or A (Tbp1 or TbpA) and transferrin binding protein 2 or B (Tbp2 or TbpB). The receptor-mediated type of iron uptake has been demonstrated to operate in serotype A bovine strains of *P. haemolytica* (26). Cells of *P. haemolytica* growing *in vitro* under iron-limited conditions express a number of iron-repressible outer membrane proteins (IROMPs) identical to those produced by cells recovered *in vivo* from infected sites in animals with pasteurellosis (9,10). Especially prominent among these proteins were those of molecular sizes 100, 77, 70 and 60 Kda (9,10). The 100 Kda protein has been identified as one of the host specific transferrin receptors in bovine isolates (26) while some of the other IROMPs had been suggested as possibly associated with the 100 Kda protein in an iron acquisition receptor complex (26). The role of the IROMPs expressed by *P. haemolytica* from lambs (10) in iron acquisition has not been elucidated, neither is it known if similar proteins are expressed by goat isolates.

P. haemolytica acquires iron from bovine host transferrin by a receptor-mediated type of mechanism. The proposal that bacteria with this type of iron acquisition mechanism may be solely dependent upon their surface receptor for iron acquisition *in vivo* (29) implies that they can only cause disease in those hosts whose transferrin is recognized by their surface receptors. *P. haemolytica* has been reported to cause disease in cattle, sheep and goats and accordingly their surface receptors would be expected to recognize these hosts' transferrins. Therefore it is important to determine whether sheep and goat isolates also possessed transferrin receptors involved in iron acquisition, to evaluate their specificities for different ruminant transferrins and to determine if there is antigenic relatedness amongst the surface receptors from the different strains causing pneumonic pasteurellosis in cattle, sheep and goats.

SUMMARY OF THE INVENTION

Transferrin receptors were identified in a collection of *Pasteurella haemolytica* (and *P. trehalosi*) strains of various serotypes and biotypes (A and T) from cattle, sheep and goats. Growth studies, binding studies and affinity isolation experiments demonstrated that these receptors had identical specificities which recognized transferrins from cattle, sheep and goats. This indicates that there are conserved regions on the receptor proteins, involved in ligand binding, which are accessible at the cell surface.

Antisera prepared against the individual purified receptor proteins (TbpA and TbpB) from a serotype A1 strain of *P. haemolytica* demonstrated considerable crossreactivity against receptor proteins from a representative selection of strains. The cross-reactivity was also observed against intact cells indicating that there are conserved immunological epitopes at the cell surface which could serve as targets for the host's immune effector mechanisms.

The present inventors have cloned, sequenced and expressed *tbpA* and *tbpB* genes encoding the transferrin receptor proteins, TbpA and TbpB (also referred to herein as Tbp1 and Tbp2, respectively), from *Pasteurella haemolytica* A1. The genes were organized in an operon arrangement of *tbpB- tbpA*. The *tbpB* gene was preceded by putative promoter and regulatory sequences, and followed by a 96 base pair intergenic sequence in which no promoter regions were found, suggesting that the two genes are coordinately transcribed. The deduced amino acid sequences of the TbpA and TbpB proteins had regions of homology with the corresponding *Neisseria meningitidis, N. gonorrhoeae, Haemophilus influenzae* and *Actinobacillus pleuropneumoniae* Lbp and Tbp proteins. The intact *tbp*B gene was expressed in a T7 expression system and the resulting recombinant TbpB protein retained the functional bovine transferrin binding characteristics. The availability of the recombinant TbpB enabled the inventors to demonstrate its specificity for ruminant transferrin, its ability to bind both the C-and N-terminal lobes of bovine transferrin, and its preference for the iron-loaded form of this protein.

The present inventors also significantly found that vaccination with a formulation containing *P. haemolytica* TbpA and TbpB provided significant protection against experimental bovine pneumonic pasteurellosis. Immunization with two doses of TbpB also provides protection.

Broadly stated, the present invention provides a purified and isolated nucleic acid molecule comprising a sequence encoding a TbpA protein, or a purified and isolated nucleic acid molecule comprising a sequence encoding a TbpB protein. The TbpA and TbpB proteins bind ruminant transferrins and function in receptor-mediated iron acquisition by *P. haemolytica* in its ruminant hosts. The TbpA protein is approximately 100kDa, and TbpB is approximately 60 kDa in size.

In an embodiment of the invention, the purified and isolated nucleic acid molecules comprise a sequence encoding a TbpA protein having the amino acid sequence as shown in Figure 22 or SEQ. ID. NO:2, or a sequence encoding a TbpB protein having the amino acid sequence as shown in Figure 24 or SEQ. ID. NO:4. In a preferred embodiment of the invention, the purified and isolated nucleic acid molecules comprise a sequence encoding a TbpA protein and having the nucleic acid sequence as shown in Figure 21 or SEQ. ID. NO:1, or a sequence encoding a TbpB protein having the nucleic acid sequence as shown in Figure 23 or SEQ. ID. NO:3.

The invention also contemplates (a) nucleic acid molecules comprising a sequence encoding a truncation of TbpA or TbpB which is unique to the protein, an analog or homolog of TbpA or TbpB or a truncation thereof, (herein collectively referred to as "TbpA related proteins" or "TbpB related proteins", respectively); (b) a nucleic acid molecule comprising a sequence

which hybridizes under high stringency conditions to the full length nucleic acid encoding TbpA or TbpB ,having the amino acid sequences as shown in Figures 22 and 24 respectively, or to a TbpA or TbpB related protein; (c) a nucleic acid molecule comprising a sequence which hybridizes under high stringency conditions to the full length nucleic acid sequence of the *tbpA* or *tbpB* genes having the sequences as shown in Figures 21 or SEQ. ID. NO:1, or Figure 23 or SEQ. ID. NO:3, respectively.

The invention further contemplates a purified and isolated double stranded nucleic acid molecule containing a nucleic acid molecule of the invention, hydrogen bonded to a complementary nucleic acid base sequence.

The nucleic acid molecules of the invention may be inserted into an appropriate expression vector, i.e. a vector which contains the necessary elements for the transcription and translation of the inserted coding sequence. Accordingly, recombinant expression vectors adapted for transformation of a host cell may be constructed which comprise a nucleic acid molecule of the invention and one or more transcription and translation elements operatively linked to the nucleic acid molecule.

The recombinant expression vector can be used to prepare transformed host cells expressing TbpA and/or TbpB, or a TbpA or a TbpB related protein. Therefore, the invention further provides host cells containing a recombinant molecule of the invention.

The invention further provides a method for preparing a novel TbpA or TbpB, and TbpA or TbpB related proteins, utilizing the purified and isolated nucleic acid molecules of the invention. In an embodiment a method for preparing TbpA or TbpB is provided comprising (a) transferring a recombinant expression vector of the invention into a host cell; (b) selecting transformed host cells from untransformed host cells; (c) culturing a selected transformed host cell under conditions which allow expression of TbpA or TbpB; and (d) isolating the recombinant TbpA or TbpB.

The invention further broadly contemplates a purified and isolated TbpA or TbpB which binds to ruminant transferrin, preferably obtained by culturing a host cell containing a recombinant expression vector of the invention. In an embodiment of the invention, a purified TbpA or TbpB is provided which has the amino acid sequence as shown in Figure 22 or Figure 24 respectively. The invention also includes truncations of the protein and analogs, homologs, and isoforms of the protein and truncations thereof (i.e., "TbpA or TbpB related proteins").

The TbpA and TbpB, or TbpA and TbpB related proteins of the invention may be conjugated with other molecules, such as proteins, to prepare fusion proteins. This may be accomplished, for example, by the synthesis of N-terminal or C-terminal fusion proteins.

The invention further contemplates antibodies having specificity against an epitope of TbpA or TbpB, or TbpA or TbpB related proteins of the invention. Antibodies may be labelled with a detectable substance and they may be used to detect the TbpA or TbpB, or TbpA or TbpB related proteins of the invention in samples.

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The invention also permits the construction of nucleotide probes which are unique to the nucleic acid molecules of the invention and accordingly to TbpA or TbpB, or TbpA or TbpB related proteins of the invention. Thus, the invention also relates to a probe comprising a sequence encoding TbpA or TbpB, or TbpA or TbpB related proteins. The probe may be labelled, for example, with a detectable substance and it may be used to select from a mixture of nucleotide sequences a nucleotide sequence coding for a protein which displays one or more of the properties of TbpA or TbpB.

The invention still further provides a method for identifying a substance which is capable of binding to TbpA or TbpB, or TbpA or TbpB related proteins, or an activated form thereof, comprising reacting TbpA or TbpB, or TbpA or TbpB related proteins, or an activated form thereof, with at least one substance which potentially can bind with TbpA or TbpB, or TbpA or TbpB related proteins, or an activated form thereof, under conditions which permit the formation of complexes between the substance and TbpA or TbpB, or TbpA or TbpB related proteins, or an activated form thereof, and assaying for complexes, for free substance, for non-complexed TbpA or TbpB or a TbpA or TbpB related proteins, or an activated form thereof. Substances which potentially can bind TbpA or TbpB, or TbpA or TbpB related proteins, include transferrins, particularly ruminant transferrins, analogs and derivatives of transferrins and antibodies against TbpA and TbpB, or TbpA or TbpB related proteins.

Still further, the invention provides a method for assaying a medium for the presence of an agonist or antagonist of the interaction of TbpA or TbpB, or TbpA or TbpB related proteins, and a substance which binds to TbpA or TbpB, or TbpA or TbpB related proteins or an activated form thereof. In an embodiment, the method comprises providing a known concentration of TbpA or TbpB, or TbpA or TbpB related proteins, with a substance which is capable of binding to TbpA or TbpB, or TbpA or TbpB related proteins and a suspected agonist or antagonist substance under conditions which permit the formation of complexes between the substance and TbpA or TbpB, or TbpA or TbpB related proteins, and assaying for complexes, for free substance, for non-complexed TbpA or TbpB, or TbpA or TbpB related proteins. In a preferred embodiment of the invention, the substance is a ruminant transferrin, analog, derivative or part thereof or an antibody against TbpA or TbpB, or TbpA or TbpB related proteins.

Substances which affect expression of TbpA or TbpB, or TbpA or TbpB related proteins, may also be identified using the methods of the invention by comparing the pattern and level of expression of TbpA or TbpB, or TbpA or TbpB related proteins of the invention, in cells in the presence, and in the absence of the substance.

The substances identified using the methods of the invention may be used in the treatment of animals, particularly ruminants infected with *P. haemolytica* and accordingly they may be formulated into pharmaceutical compositions for adminstration to ruminants, such

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as cattle, sheep and goats suffering from infection with *P. haemolytica* or exposed to infection by *P. haemolytica*.

The present inventors have demonstrated that the TbpA or TbpB, or TbpA or TbpB related proteins of the invention, are immunogenic. Therefore, the invention also relates to antibodies against the TbpA or TbpB, or TbpA or TbpB related proteins of the invention. In an embodiment, the antibodies are cross reactive against TbpA or TbpB or TbpA, or TbpB related proteins, from a wide range of serotypes of *P. haemolytica*. The antibodies may be used in the diagnosis and treatment of *P. haemloytica* infection and may be used, for example, in passive immunization to treat or prevent diseases in ruminants caused by *P. haemolytica*.

The invention further includes vaccine compositions comprising the TbpA or TbpB, or TbpA or TbpB related proteins of the invention, either alone, or in combination. The invention still further includes methods of immunizing a host, preferably a ruminant host against infection by *P. haemolytica* by administering therapeutically effective amounts of such vaccines. The present inventors have demonstrated that different strains of *P. haemolytica*, from a range of ruminants, are able to bind and utilize a range of ruminant transferrins. Thus it is contemplated that the vaccine compositions of the invention will be useful as broad spectrum vaccines suitable for immunizing a range of ruminants, such as sheep, cows and goats against infection with a wide range of *P. haemolytica* biotypes and serotypes.

The invention also contemplates the use of nucleic acid molecules of the invention encoding TbpA or TbpB, or TbpA or TbpB related proteins, in a recombinant viral vector vaccine for augmenting the immune response of a ruminant to *P. haemolytica* or for treating *P. haemolytica* infection. Recombinant viral vectors may be constructed using techniques known in the art .

Other objects, features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples while indicating preferred embodiments of the invention are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

BRIEF DESCRIPTION OF THE DRAWINGS

The invention will now be described in relation to the drawings in which:

Figure 1 is a schematic diagram of the PCR procedure (a), and the 0.8 kb PCR product amplified by Tbp1 primer and primer left (b);

Figure 2 is a restriction endonuclease map of tbp plasmids 9, 10, and 482;

~~Figure 3 is a preliminary nucleotide sequence of *P haemolytica tbpA* and *tpbB*;~~ SEQ. ID. NOS. 5 and 6

Figure 4 is the promoter region of *P haemolytica tbpB* (PHTBPB); SEQ. ID. NOS. 7, 8 and 9

Figure 5 is a blot of Southern hybridization of *P. haemolytica* genomic DNA digested with *ClaI* and probed with *tbpA* gene;

Figure 6 is a blot of Southern hybridization of *P. haemolytica* genomic DNA digested with *Hind III and Bam*HI and probed with the *tpbA* gene;

Figure 7 is a blot of Southern hybridization of *A. suis* 37114, *A. pleuropneumoniae* CM5 and shope 4074 genomic DNA digested with various restriction endonucleases and probed with *P. haemolytica tbpA*;

Figure 8 are restriction maps of the *tbpA, tbpB* regions in *P. haemolytica A1, A. pleuropneumoniae* CM5, Shope 4074, and *A. suis* 3714;

Sub H6 A

and SEQ. ID. NOS. 12, 11, and 12 Figure 9 shows an alignment of the amino acid of Tbp1 of *P. haemolytica A1*,(PHTBP) and the Tbp1 of *N.gonorrhoeae* (NGTBP1) and *N. meningitidis* (NM1);

A Sub H7

and SEQ. ID. NOS. 13, 14, and 15 Figure 10 shows an alignment of the amino acid of Tbp1 of *P. haemolytica A1*,(PHTBP) and the *A. pleuropneumoniae* serotype 1 and 7 TfbA proteins (APL, APL7);

Figure 11 is a dendogram illustrating the genetic relatedness among *P. haemolytica A1*,(PHTBP) and the Tbp1 of *N.gonorrhoeae* (NGTBP1), *N. meningitidis* (NM1) and *A. pleuropneumoniae* serotype 1 and 7 TfbA proteins (APL, APL7);

Sub H12 A

and SEQ. ID. NOS. 16, 17, 18, 19, and 20 Figure 12 is a peptide alignment between *P. haemolytica* A1 Tbp1 and TonB-dependent outer membrane receptors of *E. coli*;

Figure 13 is a blot showing T7 analysis of the *P.haemolytica* Tbp1 protein;

Figure 14 is a Western immunoblot of inner and outer membranes from *P. haemolytica* A1 and *E.coli* HB101;

Figure 15 is a Western immunoblot of inner and outer membranes from *P. haemolytica* A1 and *E.coli* HB101 using sera raised in calves to soluble antigens by vaccination with Presponse®;

Figure 16 is a blot showing the binding of labelled transferrins by iron-deficient bacterial membranes;

Sub H8

Figure 17 is an immunoblot showing isolation of receptor proteins with transferrin affinity columns;

Sub H9

Figure 18 are immunoblots showing immunological analysis of receptor proteins from different serotypes of *P. haemolytica* from bovine, sheep, and goats, where Panel A is with anti-TbpB serum and Panel B is with anti-TbpA serum;

Sub H10

Figure 19 are blots showing the binding of labelled transferrin and anti-receptor antibody by intact cells;

A Sub H11

and SEQ. ID. 1208 Figure 20 is a map of the *P. haemolytica tbp* operon (Top) and *P. haemolytica* tbp operon (Top) and regulatory sequences (Bottom); *tbpA* and *tbpB* are the genes encoding for TbpA andTbpB, respectively; p, is the putative promoter region preceding tbpB and denoted as -35 and -10 sites at the bottom;

Figure 21 and SEQ ID NO:1 show the DNA sequence of the *tbpA* gene from *P. haemolytica* strain h196;

Figure 22 and SEQ ID NO:2 show the predicted amino acid sequence of the TbpA protein from *P. haemolytica* strain h196;

Figure 23 and SEQ ID NO:3 show the DNA sequence of the *tbpB* gene from *P. haemolytica* strain h196;

Figure 24 and SEQ ID NO:4 show the predicted amino acid sequence of the TbpB protein from *P. haemolytica* strain h196;

Figure 25 is a blot showing the results of a solid-phase HRP-Tf binding assay;

Figure 26 are blots showing silver stain (Panel A), and western blot (Panel B) studies with anti-TbpA and anti-TbpB antisera from *P.haemolytica* serotype A1;

Figure 27 is a blot showing the results of cross-reactivity studies with monospecific anti-TbpA and anti-TbpB antisera from *P. haemolytica* serotype A1 against intact cells;

Figure 28 shows a gel with restriction endonuclease digestion patterns of PCR-amplified *tbpA* (Panel A) and *tbpB* (Panel B) genes from *P. haemolytica* and *P. trehalosi* strains; and

Figure 29 is a gel showing PCR amplification of variable segments of the *tbpA* (Panel A) and *tbpB* (Panel B) genes.

DETAILED DESCRIPTION OF THE INVENTION

The following standard abbreviations for the amino acid residues are used throughout the specification: A, Ala - alanine; C, Cys - cysteine; D, Asp- aspartic acid; E, Glu - glutamic acid; F, Phe - phenylalanine; G, Gly - glycine; H, His - histidine; I, Ile - isoleucine; K, Lys - lysine; L, Leu - leucine; M, Met - methionine; N, Asn - asparagine; P, Pro - proline; Q, Gln - glutamine; R, Arg - arginine; S, Ser - serine; T, Thr - threonine; V, Val - valine; W, Trp- tryptophan; Y, Tyr - tyrosine; and p.Y., P.Tyr - phosphotyrosine.

I. Nucleic Acid Molecules of the Invention

As hereinbefore mentioned, the present invention provides a purified and isolated nucleic acid molecule comprising a sequence encoding a TbpA protein, or a purified and isolated nucleic acid molecule comprising a sequence encoding a TbpB protein. The term "isolated and purified" refers to a nucleic acid substantially free of cellular material or culture medium when produced by recombinant DNA techniques, or chemical precursors, or other chemicals when chemically synthesized. An "isolated and purified" nucleic acid is also free of sequences which naturally flank the nucleic acid (i.e., sequences located at the 5' and 3' ends of the nucleic acid) from which the nucleic acid is derived. The term "nucleic acid" is intended to include DNA and RNA and can be either double stranded or single stranded.

In an embodiment of the invention, a nucleic acid molecule is provided which encodes TbpA having the amino acid sequence as shown in Figure 22 or SEQ.ID.NO:2. In another embodiment, a nucleic acid molecule is provided which encodes TbpB having the amino acid sequence as shown in Figure 24 or SEQ.ID.NO:4. In preferred embodiments of the invention, the

nucleic acid molecule is a DNA comprising the nucleotide sequence as shown in Figure 21 or SEQ.ID.NO:1, or the nucleotide sequence as shown in Figure 23 or SEQ.ID.NO:3.

The invention includes nucleic acid sequences complementary to the nucleic acid (a) encoding TbpA having the amino acid sequence as shown in Figure 22 or SEQ.ID.NO:2; (b) encoding TbpB having the amino acid sequence as shown in Figure 24 or SEQ.ID.NO:4; (c) having the sequence as shown in Figure 21 or SEQ.ID.NO:1, or in Figure 23 or SEQ.ID.NO:3. Preferably, the sequences are complementary to the full length nucleic acid sequences sequence shown in Figure 21 or SEQ.ID.NO:1, or in Figure 23 or SEQ.ID.NO:3.

The invention also includes nucleic acid molecules having substantial sequence identity or homology to the nucleic acid sequence as shown in Figure 21 or SEQ.ID.NO:1 or Figure 23 or SEQ.ID.NO:3; or encoding TbpA or TbpB proteins having substantial homology to the amino acid sequences shown in Figure 22 or SEQ.ID.NO:2, or in Figure 24 or SEQ.ID.NO:4, respectively. Homology refers to sequence similarity between sequences and can be determined by comparing a position in each sequence which may be aligned for purposes of comparison. When a position in the compared sequence is occupied by the same nucleotide base or amino acid, then the molecules are matching or have identical positions shared by the sequences.

Nucleic acid sequences having substantial homology include (a) nucleic acid sequences having at least 40-60%, preferably 60-80% most preferably 80-90% identity with the nucleic acid sequence as shown in Figure 21 or SEQ.ID.NO:1; and (b) nucleic acid sequences having at least 40-60%, preferably 60-80% most preferably 80-90% identity with the nucleic acid sequence as shown in Figure 23 or SEQ.ID.NO:3.

Another aspect of the invention provides a nucleic acid molecule, and fragments thereof having at least 15 nucleotide bases, which hybridizes to the nucleic acid molecules of the invention under hybridization conditions, preferably stringent hybridization conditions. Appropriate stringency conditions which promote DNA hybridization are known to those skilled in the art, or may be found in Current Protocols in Molecular Biology, John Wiley & Sons, N.Y. (1989), 6.3.1-6.3.6. For example, 6.0 x sodium chloride/sodium citrate (SSC) at about 45°C, followed by a wash of 2.0 x SSC at 50°C may be employed. The stringency may be selected based on the conditions used in the wash step. By way of example, the salt concentration in the wash step can be selected from a high stringency of about 0.2 x SSC at 50°C. In addition, the temperature in the wash step can be at high stringency conditions, at about 65°C.

Isolated and purified nucleic acid molecules encoding a protein having the activity of TbpA or TbpB, and having a sequence which differs from the nucleic acid sequence shown in Figure 21 or SEQ.ID.NO:1, or in Figure 23 or SEQ.ID.NO:3, respectively, due to degeneracy in the genetic code are also within the scope of the invention. Such nucleic acids should encode functionally equivalent TbpA or TbpB proteins but differ in sequence from the sequence in Figure 21 or SEQ.ID.NO:1, or in Figure 23 or SEQ.ID.NO:3, respectively, due to degeneracy in the genetic code.

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An isolated and purified nucleic acid molecule of the invention which comprises DNA can be isolated by preparing a labelled nucleic acid probe based on all or part of the nucleic acid sequence shown in Figure 21 or SEQ.ID.NO:1, or in Figure 23 or SEQ.ID.NO:3, and using the labelled nucleic acid probe to screen an appropriate DNA library (e.g. a cDNA or genomic DNA library). Nucleic acids isolated by screening of a cDNA or genomic DNA library can be sequenced by standard techniques.

An isolated and purified nucleic acid molecule of the invention which is DNA can also be isolated by selectively amplifying a nucleic acid encoding TbpA or TbpB using the polymerase chain reaction (PCR) methods and cDNA or genomic DNA. It is possible to design synthetic oligonucleotide primers from the nucleotide sequence shown in Figure 21 or SEQ.ID.NO:1, or in Figure 23 or SEQ.ID.NO:3, for use in PCR. A nucleic acid can be amplified from cDNA or genomic DNA using these oligonucleotide primers and standard PCR amplification techniques. The nucleic acid so amplified can be cloned into an appropriate vector and characterized by DNA sequence analysis. It will be appreciated that cDNA may be prepared from mRNA, by isolating total cellular mRNA by a variety of techniques, for example, by using the guanidinium-thiocyanate extraction procedure of Chirgwin et al., Biochemistry, 18, 5294-5299 (1979). cDNA is then synthesized from the mRNA using reverse transcriptase (for example, Moloney MLV reverse transcriptase available from Gibco/BRL, Bethesda, MD, or AMV reverse transcriptase available from Seikagaku America, Inc., St. Petersburg, FL).

An isolated and purified nucleic acid molecule of the invention which is RNA can be isolated by cloning a cDNA encoding TbpA or TbpB into an appropriate vector which allows for transcription of the cDNA to produce an RNA molecule which encodes a protein which exhibits TbpA or TbpB activity.

A nucleic acid molecule of the invention may also be chemically synthesized using standard techniques. Various methods of chemically synthesizing polydeoxynucleotides are known, including solid-phase synthesis which, like peptide synthesis, has been fully automated in commercially available DNA synthesizers (See e.g., Itakura et al. U.S. Patent No. 4,598,049; Caruthers et al. U.S. Patent No. 4,458,066; and Itakura U.S. Patent Nos. 4,401,796 and 4,373,071).

Determination of whether a particular nucleic acid molecule encodes a protein having TbpA or TbpB activity can be accomplished by expressing the DNA in an appropriate host cell by standard techniques, and testing the ability of the expressed protein to bind ruminant transferrins and/or mediate iron uptake. A cDNA having such activity can be sequenced by standard techniques, such as dideoxynucleotide chain termination or Maxam-Gilbert chemical sequencing, to determine the nucleic acid sequence and the predicted amino acid sequence of the encoded protein.

Regulatory elements of *tbpA* or *tbpB* can be identified using conventional techniques. The function of the elements can be confirmed by using these elements to express a reporter gene

which is operatively linked to the elements. These constructs may be introduced into cultured cells using standard procedures.

The sequence of a nucleic acid molecule of the invention may be inverted relative to its normal presentation for transcription to produce an antisense nucleic acid molecule. An antisense nucleic acid molecule may be constructed using chemical synthesis and enzymatic ligation reactions using procedures known in the art.

II. Recombinant TbpA and TbpB

The invention also contemplates a purified and isolated TbpA or TbpB protein from *Pasteurella haemolytica A1* which exhibits transferrin binding activity. In an embodiment of the invention, a purified TbpA protein is provided which has the amino acid sequence as shown in Figure 22 or in SEQ. ID. NO:2. In another embodiment of the invention, a purified TbpB protein is provided which has the amino acid sequence as shown in Figure 24 or in SEQ. ID. NO:4. Recombinant TbpB, unlike the native receptor complex, recognizes binding determinants on the N-lobe and C-lobe of transferrin.

In addition to the full length TbpA or TbpB amino acid sequences, the proteins of the present invention include truncations of TbpA or TbpB, and analogs, and homologs of TbpA or TbpB and truncations thereof as described herein. Truncated proteins may comprise peptides with at least 3 amino acid residues. The truncated proteins may have an amino group (-NH2), a hydrophobic group (for example, carbobenzoxyl, dansyl, or T-butyloxycarbonyl), an acetyl group, a 9-fluorenylmethoxy-carbonyl (PMOC) group, or a macromolecule including but not limited to lipid-fatty acid conjugates, polyethylene glycol, or carbohydrates at the amino terminal end. The truncated proteins may have a carboxyl group, an amido group, a T-butyloxycarbonyl group, or a macromolecule including but not limited to lipid-fatty acid conjugates, polyethylene glycol, or carbohydrates at the carboxy terminal end.

The proteins of the invention may also include analogs of TbpA or TbpB as shown in Figure 22 or in SEQ. ID. NO:2, or in Figure 24 or in SEQ. ID. NO:4, respectively, and/or truncations thereof as described herein, which may include, but are not limited to TbpA or TbpB (Figure 22 or in SEQ. ID. NO:2, or in Figure 24 or in SEQ. ID. NO:4), containing one or more amino acid substitutions, insertions, and/or deletions. Amino acid substitutions may be of a conserved or non-conserved nature. Conserved amino acid substitutions involve replacing one or more amino acids of the TbpA or TbpB amino acid sequence with amino acids of similar charge, size, and/or hydrophobicity characterisitics. When only conserved substitutions are made the resulting analog should be functionally equivalent to TbpA or TbpB. Non-conserved substitutions involve replacing one or more amino acids of the TbpA or TbpB amino acid sequence with one or more amino acids which possess dissimilar charge, size, and/or hydrophobicity characteristics.

One or more amino acid insertions may be introduced into TbpA or TbpB (Figure 22 or in SEQ. ID. NO:2, or in Figure 24 or in SEQ. ID. NO:4). Amino acid insertions may consist of single amino acid residues or sequential amino acids ranging from 2 to 15 amino acids in length.

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Deletions may consist of the removal of one or more amino acids, or discrete portions from the TbpA or TbpB (Figure 22 or in SEQ. ID. NO:2, or in Figure 24 or in SEQ. ID. NO:4), sequence. The deleted amino acids may or may not be contiguous. The lower limit length of the resulting analog with a deletion mutation is about 10 amino acids, preferably 100 amino acids.

The proteins of the invention also include homologs of TbpA or TbpB (Figure 22 or in SEQ. ID. NO:2, or in Figure 24 or in SEQ. ID. NO:4), and/or truncations thereof as described herein. Such TbpA or TbpB, homlogs are proteins whose amino acid sequences are comprised of the amino acid sequences of TbpA or TbpB regions from other species that hybridize under stringent hybridization conditions (see discussion of stringent hybridization conditions herein) with a probe used to obtain TbpA or TbpB.

Protein sequences having substantial homology include protein sequences having at least 40-60%, preferably 60-80%, most preferably 80-90% identity with the amino acid sequence as shown in Figure 22 (or SEQ . ID. NO:2) or Figure 24 (SEQ. ID. NO:4).

The invention also contemplates isoforms of the proteins of the invention. An isoform contains the same number and kinds of amino acids as the protein of the invention, but the isoform has a different molecular structure. The isoforms contemplated by the present invention are those having the same properties as the protein of the invention as described herein.

The present invention also includes a TbpA, TbpB or aTbpA or TbpB related proteins conjugated with a selected protein, or a selectable marker protein (see below) to produce fusion proteins. Additionally, immunogenic portions of TbpA, TbpB or aTbpA or TbpB related proteins are within the scope of the invention.

TbpA, TbpB or a TbpA or TbpB related protein of the invention are prepared using recombinant DNA methods. Accordingly, the nucleic acid molecules of the present invention having a sequence which encodes TbpA, TbpB, or a TbpA or TbpB related protein of the invention may be incorporated in a known manner into an appropriate expression vector which ensures good expression of the protein. The expression vectors are "suitable for transformation of a host cell", means that the expression vectors contain a nucleic acid molecule of the invention and regulatory sequences selected on the basis of the host cells to be used for expression, which is operatively linked to the nucleic acid molecule. Operatively linked is intended to mean that the nucleic acid is linked to regulatory sequences in a manner which allows expression of the nucleic acid.

The invention therefore contemplates a recombinant expression vector of the invention containing a nucleic acid molecule of the invention, or a fragment thereof, and the necessary regulatory sequences for the transcription and translation of the inserted protein-sequence. Suitable regulatory sequences may be derived from a variety of sources, including bacterial, fungal, viral, mammalian, or insect genes. Selection of appropriate regulatory sequences is dependent on the host cell chosen as discussed below, and may be readily accomplished by one of ordinary skill in the art.

The recombinant expression vectors of the invention may also contain a selectable marker gene which facilitates the selection of host cells transformed or transfected with a recombinant molecule of the invention. Examples of selectable marker genes are genes encoding a protein which confers resistance to certain drugs, or β-galactosidase.

The recombinant expression vectors may also contain genes which encode a fusion moiety which provides increased expression of the recombinant protein; increased solubility of the recombinant protein; and aid in the purification of the target recombinant protein by acting as a ligand in affinity purification.

Recombinant expression vectors can be introduced into host cells to produce a transformant host cell. The term "transformant host cell" is intended to include prokaryotic and eukaryotic cells which have been transformed or transfected with a recombinant expression vector of the invention. The terms "transformed with", "transfected with", "transformation" and "transfection" are intended to encompass introduction of nucleic acid (e.g. a vector) into a cell by one of many possible techniques known in the art. Suitable methods for transforming and transfecting host cells can be found in Sambrook et al. (Molecular Cloning: A Laboratory Manual, 2nd Edition, Cold Spring Harbor Laboratory press (1989)), and other laboratory textbooks.

The proteins of the invention may also be prepared by chemical synthesis using techniques well known in the chemistry of proteins such as solid phase synthesis (Merrifield, 1964, J. Am. Chem. Assoc. 85:2149-2154) or synthesis in homogenous solution (Houbenweyl, 1987, Methods of Organic Chemistry, ed. E. Wansch, Vol. 15 I and II, Thieme, Stuttgart).

N-terminal or C-terminal fusion proteins comprising TbpA, TbpB, or a TbpA or TbpB related protein of the invention conjugated with other molecules, such as proteins, may be prepared by fusing, through recombinant techniques, the N-terminal or C-terminal of TbpA, TbpB or a TbpA or TbpB related protein, and the sequence of a selected protein or selectable marker protein with a desired biological function. The resultant fusion proteins contain TbpA, TbpB or aTbpA or TbpB related protein, fused to the selected protein or marker protein.

III. Applications of the Invention

The nucleic acid molecules of the invention allow those skilled in the art to construct nucleotide probes for use in the detection of nucleic acid sequences in samples. Suitable probes include nucleic acid molecules based on nucleic acid sequences encoding at least 6 sequential amino acids from regions of the TbpA or TbpB protein as shown in Figure 22 and SEQ. ID. NO: 2, or in Figure 24 and SEQ. ID. NO: 4, respectively. For example, a suitable probe may include nucleic acid molecules of TbpA selected from nucleotide nos. 1741 to 2784 of the sequence of TbpA shown in Figure 21 and SEQ ID NO:1. A nucleotide probe may be labelled with a detectable substance such as a radioactive label which provides for an adequate signal and has sufficient half-life such as ^{32}P, ^{3}H, ^{14}C or the like. Other detectable substances which may be used include antigens that are recognized by a specific labelled antibody, fluorescent compounds, enzymes, antibodies specific for a labelled antigen, and luminescent compounds. An appropriate label

may be selected having regard to the rate of hybridization and binding of the probe to the nucleotide to be detected and the amount of nucleotide available for hybridization. Labelled probes may be hybridized to nucleic acids on solid supports such as nitrocellulose filters or nylon membranes as generally described in Sambrook et al, 1989, Molecular Cloning, A Laboratory Manual (2nd ed.). The nucleic acid probes may be used to detect genes, preferably in human cells, that encode TbpA, TbpB, or TbpA or TbpB related proteins.

TbpA, TbpB, or TbpA or TbpB related proteins of the invention can be used to prepare antibodies specific for the proteins. Conventional methods can be used to prepare the antibodies. To produce polyclonal antibodies a mammal (such as a rabbit, mouse or rat) may be immunized with TbpA,TbpB, fragments of the proteins or a mixture of the two. The immunogenicity of the protein(s) may be enhanced by adding an adjuvant to the protein mixture or by coupling the protein to an immunogenic carrier. Examples of carriers include keyhole limpet hemocyanin(KLH) and bovine serum albumin(BSA).

To produce monoclonal antibodies, antibody producing cells (lymphocytes) can be harvested from an animal (immunized as described above) and fused with myeloma cells by standard somatic cell fusion procedures thus immortalizing these cells and yielding hybridoma cells. Such techniques are well known in the art, [e.g., the hybridoma technique originally developed by Kohler and Milstein (Nature 256, 495-497 (1975)) as well as other techniques such as the human B-cell hybridoma technique (Kozbor et al., Immunol. Today 4, 72 (1983)), the EBV-hybridoma technique to produce human monoclonal antibodies (Cole et al. Monoclonal Antibodies in Cancer Therapy (1985) Allen R. Bliss, Inc., pages 77-96), and screening of combinatorial antibody libraries (Huse et al., Science 246, 1275 (1989)]. Hybridoma cells can be screened immunochemically for production of antibodies specifically reactive with the peptide and the monoclonal antibodies can be isolated. Therefore, the invention also contemplates hybridoma cells secreting monoclonal antibodies with specificity for TbpA or TbpB, or TbpA or TbpB related proteins, as described herein.

The term "antibody" as used herein is intended to include fragments thereof which also specifically react with a protein, or peptide thereof, having the activity of TbpA or TbpB. Antibodies can be fragmented using conventional techniques and the fragments screened for utility in the same manner as described above. For example, $F(ab')_2$ fragments can be generated by treating antibody with pepsin. The resulting $F(ab')_2$ fragment can be treated to reduce disulfide bridges to produce Fab' fragments. Multivalent antibodies may be prepared by fusing two or more $F(ab')_2$ or Fab' fragments. For example, a multivalent antibody may contain one $F(ab')_2$ fragment specific for TbpA and one F(ab')2 fragment specific for TbpB.

Chimeric antibody derivatives, i.e., antibody molecules that combine a non-ruminant animal variable region and a ruminant constant region are also contemplated within the scope of the invention. Chimeric antibody molecules can include, for example, the antigen

binding domain from an antibody of a mouse, rat, or other species, with bovine constant regions. Conventional methods may be used to make chimeric antibodies containing the immunoglobulin variable region which recognizes the gene product of novel *Tbp* genes of the invention (See, for example, Morrison et al., Proc. Natl Acad. Sci. U.S.A. 81,6851 (1985); Takeda et al., Nature 314, 452 (1985), Cabilly et al., U.S. Patent No. 4,816,567; Boss et al., U.S. Patent No. 4,816,397; Tanaguchi et al., European Patent Publication EP171496; European Patent Publication 0173494.

Antibodies specifically reactive with TbpA, TbpB, or TbpA or TbpB related proteins, or derivatives thereof, such as enzyme conjugates or labeled derivatives, may be used as probes to detect TbpA, TbpB, or TbpA or TbpB related proteins in samples such as tissues and cells, for example they may be used in any known immunoassays which rely on the binding interaction between an antigenic determinant of TbpA, TbpB, or TbpA or TbpB related proteins and the antibodies. Examples of such assays are radioimmunoassays, enzyme immunoassays (e.g.ELISA), immunofluorescence, immunoprecipitation, latex agglutination, hemagglutination, and histochemical tests. Thus, the antibodies may be used to detect and quantify TbpA, TbpB, or TbpA or TbpB related proteins in a sample. In an embodiment, the antibodies are cross reactive against TbpA or TbpB or TbpA, or TbpB related proteins, from a wide range of serotypes of *P. haemolytica*. When used as probes the antibodies are usually labelled by techniques known in the art.

The antibodies of the present invention may also be used in the diagnosis and treatment of *P. haemloytica* infection. In one embodiment, the antibodies are used in passive immunization to treat or prevent diseases in ruminants caused by *P. haemolytica*. In such a case, a mixture of antibodies or a multivalent antibody may be used.

The invention still further provides a method for identifying a substance which is capable of binding to TbpA or TbpB, or TbpA or TbpB related proteins, or an activated form thereof, comprising reacting TbpA or TbpB, or TbpA or TbpB related proteins, or an activated form thereof, with at least one substance which potentially can bind with TbpA or TbpB, or TbpA or TbpB related proteins, or an activated form thereof, under conditions which permit the formation of complexes between the substance and TbpA or TbpB, or TbpA or TbpB related proteins, or an activated form thereof, and assaying for complexes, for free substance, for non-complexed TbpA or TbpB or a TbpA or TbpB related proteins or an activated form thereof. Substances which potentially can bind TbpA or TbpB, or TbpA or TbpB related proteins, include transferrins, particularly ruminant transferrins, analogs and derivatives of transferrins and antibodies against TbpA and TbpB, or TbpA or TbpB related proteins.

Still further, the invention provides a method for assaying a medium for the presence of an agonist or antagonist of the interaction of TbpA or TbpB, or TbpA or TbpB related proteins, and a substance which binds to TbpA or TbpB, or TbpA or TbpB related proteins or an activated form thereof. In an embodiment, the method comprises providing a known concentration of TbpA or TbpB, or TbpA or TbpB related proteins, with a substance which is

capable of binding to TbpA or TbpB, or TbpA or TbpB related proteins and a suspected agonist or antagonist substance under conditions which permit the formation of complexes between the substance and TbpA or TbpB, or TbpA or TbpB related proteins, and assaying for complexes, for free substance, for non-complexed TbpA or TbpB, or TbpA or TbpB related proteins. In a preferred embodiment of the invention, the substance is a ruminant transferrin, analog, derivative or part thereof ,or an antibody against TbpA or TbpB, or TbpA or TbpB related proteins.

Substances which affect expression of TbpA or TbpB, or TbpA or TbpB related proteins, may also be identified using the methods of the invention by comparing the pattern and level of expression of TbpA or TbpB, or TbpA or TbpB related proteins of the invention, in cells in the presence, and in the absence of the substance.

The substances identified using the methods of the invention may be used in the treatment of animals, particularly ruminants infected with *P. haemolytica* and accordingly they may be formulated into pharmaceutical compositions for adminstration to ruminants, such as cattle, sheep and goats suffering from infection with *P. haemolytica,* or exposed to infection by *P. haemolytica.*

The substances may be formulated into pharmaceutical compositions for adminstration to subjects in a biologically compatible form suitable for administration *in vivo*. By "biologically compatible form suitable for administration *in vivo*" is meant a form of the substance to be administered in which any toxic effects are outweighed by the therapeutic effects. The substances may be administered to living organisms including humans, and animals. Administration of a therapeutically active amount of the pharmaceutical compositions of the present invention is defined as an amount effective, at dosages and for periods of time necessary to achieve the desired result. For example, a therapeutically active amount of a substance may vary according to factors such as the disease state, age, sex, and weight of the individual, and the ability of antibody to elicit a desired response in the individual. Dosage regima may be adjusted to provide the optimum therapeutic response.

The active substance may be administered in a convenient manner such as by injection (subcutaneous, intravenous, etc.), oral administration, inhalation, transdermal application, or rectal administration. Depending on the route of administration, the active substance may be coated in a material to protect the compound from the action of enzymes, acids and other natural conditions which may inactivate the compound.

The compositions described herein can be prepared by per se known methods for the preparation of pharmaceutically acceptable compositions which can be administered to subjects, such that an effective quantity of the active substance is combined in a mixture with a pharmaceutically acceptable vehicle. Suitable vehicles are described, for example, in Remington's Pharmaceutical Sciences (Remington's Pharmaceutical Sciences, Mack Publishing Company, Easton, Pa., USA 1985). On this basis, the compositions include, albeit not

exclusively, solutions of the substances in association with one or more pharmaceutically acceptable vehicles or diluents, and contained in buffered solutions with a suitable pH and iso-osmotic with the physiological fluids.

TbpA or TbpB, and/or TbpA or TbpB related proteins may be used as vaccines for the prophylaxis and treatment of various infectious diseases in animals. Infectious diseases contemplated by the invention include infections caused by *P. haemolytica*. such as bovine pneumoniae in cattle and systemic disease and pneumonia in sheep. In addition, vaccines according to the present invention may be used for the prophylaxis and treatment of infections caused by other Pasteurella spp. One example is the prophylaxis and treatment of *Pasteurella multocida* in cattle,swine and poultry including respiratory and systemic infections such as Hemorrhagic septicemia and bovine mastisis. The vaccines can be intended for administration to various animals, preferably ruminants, including cattle, sheep, and goats.

The present inventors have demonstrated that different strains of *P. haemolytica*, from a range of ruminants, are able to bind and utilize a range of ruminant transferrins. Thus it is contemplated that the vaccine compositions of the invention will be useful as broad spectrum vaccines suitable for immunizing a range of ruminants, such as sheep, cows and goats against infection with a wide range of *P. haemolytica* biotypes and serotypes.

The vaccine compositions comprise the TbpA ,TbpB, and/or TbpA or TbpB related proteins, either alone, or in combination. The vaccine compositions may contain any combination of the described proteins or immunogenic fragments thereof. Further, the composition may contain Tbp proteins isolated from one or more biotypes or serotypes of *P. haemolytica* or other microorganisms. Recombinant proteins comprising TbpA, TbpB, and/or TbpA or TbpB related proteins are preferably employed in the vaccine compositions of the invention. In a preferred embodiment, one or more of recombinant TbpA or TbpB, and TbpA or TbpB related proteins of the invention, are used in the vaccine compositions. In another embodiment of the invention, the vaccine composition consists of purified and isolated TbpA and TbpB, preferably recombinant TbpA and TbpB.

The vaccine of the invention contains an immunologically effective amount of one or more of TpbA, TbpB, TbpA related protein, and TbpB related protein. The optimum amounts of the proteins depends on the nature of the infection against which protection is required, the characteristics of the animals to be protected, and other factors known to persons skilled in the art.

In addition to the TpbA, TbpB, TbpA related protein, and/or TbpB related protein, the vaccine may comprise an immunologically acceptable carrier such as aqueous diluents, suspending aids, buffers, excipients, and one or more adjuvants known in the art. Suitable adjuvants include aluminum hydroxide, Freund's adjuvant (complete or incomplete), bacteria such as *Bordetella pertussis* or *E. coli* or bacterium derived matter, immune stimulating complex (iscom), oil, sapronin, oligopeptide, emulsified paraffin-Emulsigen™ (MVP Labs, Ralston,

Nebraska), L80 adjuvant containing $AL(OH)_3$ (Reheis, New Jersey), Quil A (Superphos), or other adjuvants known to the skilled artisan. Preferably, the adjuvant is L80 adjuvant containing $AL(OH)_3$ (Reheis, New Jersey) and Quil A (Superphos). The vaccine can be incorporated into a liposome system which will allow the slow release of the TbpA and/or TbpB protein in the recipient. The vaccine may also contain preservatives such as sodium azide, thimersol, gentamicin, neomycin, and polymyxin.

The vaccine may be a multivalent vaccine and additionally contain other immunogens of *P. haemolytica* or immunogens related to other diseases in a prophylactically or therapeutically effective manner. For example, the vaccine composition of the inventon may consist of *P. haemolytica* leukotoxin and TbpB.

The vaccines of the invention may be administered in a convenient manner, such as intravenously, intramuscularly, subcutaneously, intraperitoneally, intranatally or orally. Preferably the vaccine is administered intramuscularly or subcutaneously.

The dosage will depend on the nature of the infection, on the desired effect and on the chosen route of administration, and other factors known to persons skilled in the art.

The invention also contemplates the use of recombinant viral vector vaccines and recombinant bacterial vector vaccines containing nucleic acid molecules of the invention encoding TbpA, TbpB, TbpArelated protein, and/or TbpB related protein, for the treatment and/or prophylaxis of *P. haemolytica* infection. In such systems, the TbpA or TbpB proteins are synthesized *in vivo* in the recipient from the exogenous nucleic acid molecules in the vaccine. The recombinant viral or bacterial vectors may be constructed using techniques known in the art, and as described herein. Examples of bacterial systems include *E.coli* and *Salmonella spp.*

The following non-limiting examples are illustrative of the present invention:

Examples

EXAMPLE 1

The following materials and methods were used in the studies described in the example:

MATERIALS AND METHODS

BACTERIAL STRAINS AND CLONING VECTORS

P. haemolytica strains were provided by Dr. P. Shewen, Department of Veterinary Microbiology and Immunology (VMI), University of Guelph, and were originally obtained from Dr. E. Biberstein, University of California, Davis, Dr. G. Frank, USDA, Ames, Iowa, and Dr. W. Donachie, Moredun Research Institute, Edinburgh, U.K. *Actinobacillus suis* strain 3714, *A. pleuropneumoniae* strains CM5 and Shope 4074 were provided by Dr. S. Rosendal, VMI. *E. coli* strains HB101 and TG-1 were provided by Dr. R. Lo, Department of Microbiology, University of Guelph, and were used as recipient strains for cloning experiments. *E. coli* strain JM109 (DE3) was provided by Dr. C. Whitfield, Department of Microbiology, University of Guelph.

Pasteurella and *Actinobacillus* strains were maintained on sheep's blood agar and cultured in brain heart infusion broth (BHIB), (Difco Labs, Detroit, Michigan). *E. coli* HB101

was grown on Luria-Bertaini plus thymidine (LT), supplemented with ampicillin (Sigma Chemical Co., St. Louis, Missouri) at 100 mg/L for selection of recombinant plasmids. Similarly, *E. coli* TG-1 and JM109 (DE3) were grown on Davis minimal medium with ampicillin. Iron-depleted conditions were prepared by adding the iron chelator ethylenediamine-di(*o*-hydroxyphenylacetic) acid (EDDA) (Sigma) to a final concentration of 100μM. Iron-depleted conditions were prepared by adding $FeCl_3$ to 1 mM.

The plasmid pBR322, bacteriophage vectors M13/mp18 and M13/mp19 were used as previously described (Lo and Cameron, 1986; Lo *et al.*, 1985; and Lo *et al.*, 1987). The pBluescript vector was obtained from Stratagene (La Jolla, California). The recombinant Clone 482 was provided by Dr. A. Schryvers, Department of Microbiology, University of Calgary.

ENZYMES, CHEMICALS AND ANTISERA

Restriction endonucleases and DNA modifying enzymes were purchased from Bethesda Research Laboratories (BRL) (Burlington, Ontario) or Pharmacia Chemicals Incorporated (Dorval, Quebec) and were used as described by the manufacturers. Radioisotopes were purchased from ICN Biomedical (Montreal, Quebec) or Amersham Laboratories (Oakville, Ontario).

Goat anti-rabbit immunoglobulin G-alkaline phosphatase conjugate and immunodectection reagents were purchased from Bio-Rad Laboratories (Mississauga, Ontario). Goat anti-bovine immunoglobulin G-Alkaline phosphatase conjugate was purchased from Jackson Immunoresearch (West Grove, PA). Rabbit anti-autologous antiserum and bovine anti-Presponse antisera were obtained from Dr. P. Shewen, Department of Veterinary Microbiology and immunology, University of Guelph. The rabbit "anti-autologous" antiserum was raised against the soluble antigens of *P. haemolytica* A1 cultured in RPMI 1640 supplemented with that rabbit's own serum. It is important to note that RMPI 1640 is an iron poor medium.

DNA METHODS

a) **Chromosomal DNA isolation**

Chromosomal DNA was isolated from bacterial cells according to the method of Marmer (1961). Bacteria were inoculated into 250 ml of the appropriate medium and grown overnight at 37°C with 150 rpm shaking. The following day, the cells were pelleted by centrifugation at 4,000 x g in a GSA rotor in a Sorvall RC5-B refrigerated centrifuge (Dupont Instruments, Mississauga, Ontario) for 10 min. The pellet was suspended in 8 ml of a 0.6 M sorbitol, 0.05 mM Tris-HCI (pH 8.0), 0.05 M EDTA solution. Lysozyme (Sigma) was added to a final concentration of 3 mg/ml and the sample was incubated for 30 min on ice. Two ml of lytic solution (0.5% SDS, 0.05 M EDTA, 0.05 M Tris-Cl [pH 8.0]) and 3 mg/ml of proteinase K (Sigma) solution were added to the sample, which was then incubated for 4 h in a 37°C water bath, followed by incubation at 56°C.

The suspension was extracted with an equal volume of phenol (Gibco/BRL) saturated with TE buffer (0.05 M Tris-HCl [pH 7.5], 0.001 M EDTA) and shaken at 30-50 rpm for 45 min.

The phenol and aqueous phases were separated by centrifugation at 12,000 x g at 5°C for 10 min in a SS34 rotor. The supernatant was collected by a cut off wide mouth pasteur pipette and the DNA was precipitated with 2-3 volumes of ice-cold 95% ethanol. The strands of DNA were spooled onto a glass rod and dissolved in a small volume of 0.1 X SSC (1X SSC contains 0.15 M NACl, 0.015 M sodium citrate).

The DNA was then treated with RNase to a final concentration of 10µg/ml and incubated at 37°C for 30 min. The DNA was again precipitated by 2-3 volumes of cold 95% ethanol, spooled onto a glass rod and dissolved in 1X SSC. Samples were stored at 4°C.

b) Restriction endonuclease digestion and ligation

Plasmid and bacteriophage vectors were digested with the appropriate restriction endonucleases according to manufacturer's instructions. Vector and insert DNA were mixed to a final volume of 5µl and were ligated with 0.5 units of T4 DNA ligase. Ligation mixtures were either incubated for 3-4 hours at room temperature, or overnight at 14°C prior to tranformation into *E. coli* cells.

c) Preparation of competent *E. coli* cells

Transformation was used to introduce plasmid and bacteriophage DNA into competent *E. coli* cells (Mandel and Higa, 1970; Lederberg and Cohen *et al.*, 1972). *E. coli* strains to be transformed were grown overnight in LT broth at 37°C with 150 rpm shaking. The following day, a 1/40 subculture in 20 ml of the same medium was prepared and grown for an additional 60 min at 37°C with 75 rpm shaking. The cells were collected by centrifugation at 3,000 x g in an SS34 rotor and resuspended in 10 ml sterile ice-cold 50 mM $CaCl_2$. The suspension was incubated for 30 min. on ice, then the cells were collected by centrifugation and resuspended in 2 ml sterile ice-cold mM $CaCl_2$. The competent cells could then be stored at 4°C and used for up to 3 days.

For transformation, 0.2 ml of the competent cells were mixed with the DNA sample and incubated for 30 min on ice. The cells were heat-shocked for 2 min at 42°C and then 0.2 ml of LT broth was added. The cells were incubated at 37°C for 15 min then plated onto LT plates containing appropriate antibiotics and incubated overnight at 37°C.

d) Large-scale plasmid isolation

Large-scale plasmid isolation was performed according to the procedure of Clewell and Helinski (1969) with modifications. *E. coli* carrying the plasmid was inoculated into 250 ml LT broth containing ampicillin and grown overnight at 37°C with 150 rpm shaking. The following day, chloramphenicol (Sigma) was added to a final concentration of 25 mg/l and the culture was grown for a further 4-6 h. The cells were collected by centrifugation at 4,000 x g for 10 min in a GSA rotor. The cell pellet was resuspended in 4 ml of an ice-cold solution containing 25% sucrose and 0.05 M Tris HCl (pH 8.0), then 1 ml of a fresh 10 mg/ml lysozyme (Sigma) solution was added. The mixture was incubated in a 37°C waterbath for 30 min, placed on ice for 5 min and then 2 ml of 0.25 M EDTA (pH 8.0) was then added. After a further 5 min incubation on ice, 5

ml of a lytic solution (0.05 M Tris-HCl [pH 8.0], 0.0625 M EDTA and 2% Triton X-100) were added. The mixture was returned to the 37°C waterbath for 5-15 min until cell lysis was complete. The mixture was then centrifuged for 30 min at 27,000 x g and the clear lysate was transferred to a clean test tube, the lysate was mixed with solid CsCl (Boehringer Mannheim, Laval, Quebec) at 1 g/ml to a total of 4.5 ml. One hundred µl of ethidium bromide (10 mg/ml) were then added in 4.5 ml of the sample. The centrifuge tube was heat sealed and the sample was centrifuged at 240,000 x g for a minimum of 9 h at 15°C in a Beckman VTi65 vertical rotor.

Plasmid DNA was recovered by puncturing the top and bottom of the centrifuge tube and collecting the lower of two bands in the tube. To extract the ethidium bromide from the sample, the plasmid DNA solution was mixed with an equal volume of Cs/Cl satured n-butanol. After allowing the phases to separate, the upper layer containing n-butanol and ethidium bromide was removed. This process was repeated three times. Following ethidium bromide extraction, the lower aqueous phase was dialyzed to remove the CsCl. Dialysis tubing (Fisher) with a molecular cutoff of 10 kDa was prepared by boiling 2 x 15 min in 0.1 M Na bicarbonate and 1 x 15 min in 0.25 M EDTA (pH 7.5) and was stored at 4°C in 50% ethanol and 1mM EDTA. Prior to dialysis, the tubing was rinsed in dH_20 and then filled with the plasmid DNA solution. The DNA was dialyzed for 24 h in 4 x 1L of dialysis buffer (0.01 M Tris-HCl [pH 7.5 at 4°C], and 0.001 M EDTA) at 4°C. The sample was stored at -20°C.

Alternatively, the Flexi-prep kit from Pharmacia (Quebec City, Quebec) was used for small-scale plasmid preparation. This method involved a standard alkaline cell lysis, including RNase treatment and isopropanol precipitation (Birnboim and Doly, 1979; Isch-Horowicz and Burke, 1981). The plasmid DNA was purified and concentrated using a silica matrix (Sephaglas FP™) in guanidine hydrochloride.

e) Radiolabelling of DNA probes by random priming

DNA fragments were labelled with [α-^{32}P] dATP (3,000 Ci/mmol, ICN) using the random primer DNA labelling system of GIBCO/BRL. This labelling system is based on the method of Feinberg and Vogelstein (1983), with modifications (Feinberg and Vogelstein, 1984). The sample (25 ng of DNA in 10 µl of H_2O) was denatured by boiling for 5 min, then immediately cooled on ice. While still on ice, the following reagents were added: 2µl of each of dCTP, dGTP and dTTP, 15 µl of random primer buffer, 4 µl [α-^{32}P]dATP and H_2O to 49µl. The sample was mixed briefly and 3 units of Klenow Fragment was added. The reaction mixture was incubated for 1 h at 25°C and terminated by the addition of 5 µl of stop buffer.

The radiolabelled DNA was separated from unincorporated radionucleotides by gel filtration through a mini Sephadex G-50 column. The column was prepared in a Pasteur pipette plugged with glass wool and was equilibrated with TE buffer prior to addition of the radiolabelled sample. The migration of DNA through the column was monitored using a Geiger counter (Mini-Instruments Ltd., Essex, England). The first peak of radioactivity corresponded to

the labelled DNA, while the second peak corresponded to the unincorporated [^{32}P]-dATP. The DNA probe was denatured by boiling for 5 min before being added to the hybridization solution.

f) Agarose gel electrophoresis and Southern hybridization

Agarose gels were prepared by adding TAE buffer (40 mM Tris [pH 7.9], 1mM EDTA) to electrophoresis grade agarose powder (regular or low-melting point; Sigma) to a final concentration of 0.7% to 1%. The agarose gel was electrophoresed in a horizontal flatbed gel apparatus (Tyler Research, Edmonton, Alberta).

DNA samples were mixed with 1/2 volume of tracking dye (50% glycerol, 0.1% ladder (Gibco/BRI), or lambda DNA (Pharmacia) digested with *Hind*III was used as a molecular standard. A running buffer of TAE supplemented with 1 μg/ml of ethidium bromide was used. Samples were initially electrophoresed at 100V for 5 min, then the voltage was reduced to 10-12V for overnight electrophoresis. After electrophoresis, the samples were viewed with a medium range ultraviolet transilluminator and photographed using Polaroid type 57 black and white film (Sharp *et al.*, 1973; Hayward, 1972).

For Southern hybridization, the agarose gel was immersed in 0.25 M HCl for 15 min to depurinate the DNA. The gel was transferred to an alkaline solution consisting of 0.5 M NaOH and 1.5 M NaCl for 15 min, then neutralized in a solution of 0.5 M Tris-HCl (pH 7.5), 1.5 M NaCl for 30 min. The DNA was transferred to a nitrocellulose membrane (Schleicher and Shuell, Willowdale, Ontario) by electrophoretic transfer in a semi-dry blotting apparatus (Tyler Research) in 20X SSPE buffer (3.6 M NaCl, 0.2 M Na_2PO_4 [pH 7.0], 0.02 M Na_2EDTA, 0.16 M NaOH) at a constant current of 150 mA for 30 minutes (Wahl *et al.*, 1979; Southern, 1975).

After electrophorectic transfer, the nitrocellulose membrane was washed in 2X SSPE buffer for 10 min, and the DNA was cross-linked by a UV Cross-linker (Stratagene). The membrane was prehybridized in a sealed plastic bag containing a solution of 25% (low stringency) or 50% (high stringency) formamide (Gibco/BRL) in 0.1% glycine, 5X BFP (100X BFP contains 2% w/v bovine serum albumin, Ficoll and polyvinyl pyrrolidine-40), 5X SSPE buffer and 0.1 mg/ml sonicated, boiled salmon sperm carrier DNA. The sealed bags were placed in a 42°C shaking waterbath where the membranes were allowed to prehybridize for at least an hour. The prehybridization buffer was then discarded and replaced with hybridization buffer (10% dextran sulphate, 5X SSPE, 5X BFP, 0.1% SDS, 0.1 mg/ml carrier DNA and 25% or 50% formamide) containing the boiled, radiolabelled DNA probe. The bags were placed in a 42°C shaking waterbath where the membranes were hybridized overnight.

After hybridization, the nitrocellulose membrane was removed from the plastic bag and washed 4 x 10 min in either high stringency (5X SSPE, 0.1% SDS) or low stringency (2X SSPE, 0.1% SDS) wash buffer in a 42°C shaking waterbath. The membrane was air-dried, placed on Whatman filter paper, covered with plastic wrap and exposed to X-ray film (Cronex, Willingmington, Delaware) at -20°C for 104 days until the desired exposure was obtained. The exposure time was determined by measuring the intensity of the radioactive signal using a

Geiger counter. Autoradiographs were developed in Kodak GBX rapid developer (Eastman Kodak, Rochester, New York) for 2 min. The developing reaction was stopped by immersing the film in 2.5% acetic acid for 1 min and fixed for 2 min in Kodak GBX fixer (Eastman Kodak).

g) Southern colony blot

A master template of bacterial colonies grown on LT plus ampicillin was grown overnight at 37°C. The colonies on the master plate were duplicated onto a nitrocellulose membrane overlaid on an LT plus ampicillin plate and grown for 2-3 h at 37°C. The membrane was then overlaid on Whatman filter paper soaked in a 0.5 M NaOH, 1.5 M NaCl solution and incubated at room temperature (RT) for 5 min to lyse the cells. The nitrocellulose membrane was then transferred to Whatman filter paper soaked in a 0.5 M Tris=HCl (pH 7.5), 1.5 M NaCl solution and incubated for 5 min at RT to neutralize the membrane. The membrane was transferred to Whatman filter paper soaked in 95% ethanol and sprayed with 95% ethanol to precipitate the DNA. The DNA on the membrane was cross-linked in a UV cross-linker (Stratagene), then prehybridized and hybridized as described above.

h) Polymerase chain reaction (PCR)

PCR reactions were carried out in thin-walled 500 µl GeneAmp microfuge tubes in a Perkin-Elmer Cetus 480 DNA Thermal Cycler, using the Perkin-Elmer Cetus PCR core reagent kit which included deoxynucleotides triphosphates, $MgCl_2$, reaction buffer and Ampli-Taq DNA polymerase (Perkin-Elmer Cetus). Amplification reactions were performed according to the method of Saiki *et al.* (1988), with modifications by Perkin-Elmer Cetus. PCR reactions were performed in 100 µl mixtures containing 1X reaction buffer (0.5 M KCl, 0.1 M Tris-HCl [pH 9.0]), 0.2 mM of each of dNTP, 0.4 µM primer, 5 µg of template, 15 mM $MgCl_2$ and 2.5 units of Ampli-Taq enzyme. The reaction mixture was heated at 95°C for 2 minutes to denature the template DNA. Then 30 cycles of denaturation, annealing and extension followed with temperatures and times of 95°C (1 min) 52°C (1 min) and 72°C (2 min) respectively. The fastest available transitions between temperatures (ramp time of 0.01s) were used. A negative control which did not contain template DNA was included in each PCR run.

After amplification, the PCR products were examined by agarose gel electrophoresis. PCR products were purified by electrophoretic separation through a low-melting point agarose gel followed by excision of required DNA fragments. The DNA products were purified from the agarose using a glass-bead matrix purification kit (GENECLEAN).

i) Purification of DNA fragments from agarose gels

DNA fragments were purified from agarose gels using the GENECLEAN kit from Bio/Can Scientific (Mississauga, Ontario). The GENECLEAN purification process is based on the procedure by Vogelstein and Gillespie (1979). The gel slice containing the fragment was excised from the gel using a razor blade and placed in a 1.5 ml Eppendorf centrifuge tube. An equal volume of stock NaI solution was added and the sample was incubated for 5 min in a 55°C waterbath until the agarose had completely melted.

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GLASSMILK (Bio/Can Scientific) was added to the sample at a volume of 5 µl for 5µg or less of DNA and the mixture was incubated on ice for 5 min. The silica matrix was then collected by centrifugation at 16,000 x g for 10 sec and resuspended in 600 µl of NEW wash buffer (Bio/Can Scientific). The pellet was washed with NEW buffer a total of three times. After the final wash, the silica matrix was resuspended in 10µl of TE buffer and incubated for 5 min at 55°C. The sample was then centrifuged and the TE recovered, avoiding the silica matrix pellet. Samples were stored at -20°C.

j) DNA dideoxy sequencing

DNA fragments were sequenced either by cloning into M13 mp18/mp19 bacteriophage vectors (single-stranded sequencing) or directly from recombinant plasmids (double-stranded sequencing) using the Pharmacia T7-sequencing kit as described by the manufacturer. The Pharmacia T7-sequencing kit procedure is based on the method outlined by Sanger *et al.* (1977).

For single stranded sequencing, DNA fragements were cloned into the M13 mp18/mp19 bacteriophage vector and transformed into competent *E. coli* TG-1 cells. Recombinant phage "plaques", which appeared white due to the loss of β-galactosidase production, were selected. Each plaque was inoculated into 10 ml of LT broth seeded with 0.1 ml overnight culture of *E. coli* TG-1 grown in Davis minimal medium and incubated 4-5 h at 37°C with 75 rpm shaking. The sample was centrifuged at 12,000 x g for 10 min to remove the *E. coli* cells. The phage were precipitated from the culture supernatant by the addition of 1/4 volume of 20% polyethylene glycol (8,000 MW; Sigma), 2.5 M NaCl and incubated for 30 min on ice. Precipitated phage were recovered by centrifugation in a microfuge at 12,000 x g for 10 min. The pellet was then resuspended in 0.6 ml of phage buffer (0.1 M Tris-HCl [pH 8.0], 0.001 M EDTA, 0.3 M NaCl).

The phage DNA was extracted with 0.5 ml of phenol (Gibco/BRL) saturated with TE buffer. The phenol and aqueous phases were separated by centrifugation at 14,000 x g for 10 min. The aqueous phase was extracted with 1:1 phenol:chloroform and finally with chloroform. The phage DNA was then precipitated with 1/10 volume 3 M sodium acetate (pH 7.0) and 2 volumes of cold 95% ethanol and incubated at -20°C overnight. Precipitated DNA was collected by centrifugation at 14,000 x g for 10 min. The aqueous phase was extracted with 1:1 phenol:chloroform and finally with chloroform. The phage DNA was then precipitated with 1/10 volme 3 M sodium acetate (pH 7.0) and 2 volumes of cold 95% ethanol and incubated at -20°C overnight. Precipitated DNA was collected by centrifugation at 14, 000 x g for 10 min in a 4°C microfuge. The pellet was air-dried and resuspended in 50 µl of TE buffer and was used for sequencing. The DNA was annealed to either the universal M13 primer or specific primers in the presence of annealing buffer (Pharmacia T7 sequencing kit) by incubation at 65°C for 10 min, then room temperature for 10 min prior to the sequencing reactions.

For double-stranded sequencing, the plasmid template was prepared using the procedure outlined in the Pharmacia T7 sequencing kit protocol, with modifications. Plasmid

DNA was adjusted to 1.5-2.0 µg/32 µl and denatured by the addition of 12 µl of 2M NaOH for 1 min. Denaturation was terminated by the addition of 11 µl of 3 M sodium acetate (pH 5.0). The DNA was precipitated with 7 µl of dH_2O and 120 µl of ice-cold absolute ethanol and incubated at -20°C overnight.

The DNA was collected by centrifugation at 14, 000 x g in a 4°C microfuge for 10 min. The pellet was washed with 100 µl of ice-cold 70% ethanol, centrifuged and dried under vacuum. The sample was resuspended in 5 µl of dH_2O and mixed with 5 µl of primer and 2 µl of annealing buffer. The mixture was incubated at 65°C for 5 min, 37°C for 10 min and 5 min at RT prior to sequencing.

Either [^{32}P]dATP or [^{35}S]dATP (specific activity of 3000 Ci/mmol) were used in the sequencing reactions. For short autoradiography exposure time, [^{32}P]dATP was used. For superior resolution, [^{35}S]dATP was used. Oligonucleotide primers were synthesized on an Applied Biosystems International 391 PCR-Mate DNA synthesizer and purified according to the manufacturer's instructions. The primers were quantitated by measuring the optical density at 260 nm prior to use.

For [^{32}P]dATP sequencing, the sequencing gel consisted of 18 g of urea (ICN, Montreal, Quebec), 3.75 ml of 10X TBE buffer (1 M Tris [pH 8.3], 0.02 M EDTA, and 0.865 M boric acid) and 7.5 ml of 40% acrylamide (19:1 ratio of acrylamide:bisacrylamide, Bio-Rad) made up to 38 ml with dH_2O. The solution was stirred until the urea was dissolved and then 0.23 ml of 10% ammonium persulfate (Sigma) and 10 µl of TEMED (N, N, N'N'-Tetramethylethylenediamine; Sigma) (0.01% final concentration) were added for polymerization.

For [^{35}S]dATP sequencing the gel consisted of 16.8 g of urea (ICN), 4.8 ml of 10X TBE buffer and 4 ml of a modified acrylamide solution ("Long Ranger", J.T. Baker, Phillipsburg, New Jersey). The solution was made up to 40 ml with dH_2O and polymerized with 200 µl of ammonium persulfate (Sigma) and 20 µl of TEMED (Sigma).

The running buffer consisted of 1X TBE. The sequencing gel was run at 40 W/gel constant power for 2-6 hr. [^{35}S]dATP sequencing gels were transferred onto a sheet of Whatman filter paper and dried under vacuum at 80°C for 45 min. Both types of sequencing gels were exposed to Cronex 4 X-ray film (Cronex for 18-48 h at -20°C.

IV. PROTEIN METHODS

a) Isolation of inner and outer membranes

Inner and outer membrane preparations were prepared from *E. coli* and *P. haemolytica* A1 by the procedure of Hancock and Carey (1979), with modifications (Lo *et al.*, 1991). Bacteria were grown in 250 ml of the appropriate medium at 37°C overnight. The cells were collected by centrifugation at 4,000 x g, washed twice in 0.01 M Tris-HCl (pH 6.8) and resuspended in 7.5 ml of a cold Sucrose-Tris solution containing 20% sucrose, 0.01 M Tris-HCl (pH 6.8), lysozyme (1mg/ml), DNase (50µg/ml), and RNase (100 µg/ml). The cells were lysed by

French pressure cell three times at 16,000 - 18,000 psi at 4°C. The sample was then centrifuged at 1,085 x g for 5 min to remove unlysed cells.

The supernatant was layered onto a 70:52:sample:12% sucrose gradient which consisted of 14 ml of the 70 and 52% sucrose followed by 5 ml of the sample lysate and 4-5 ml of 12% sucrose. The gradient was centrifuged in a swinging bucket rotor at 80,000 x g for 16-18 h at 4°C. The inner and outer membrane fractions were collected by aspiration. The inner membrane fraction was located between the 12% and 52% sucrose regions and had a yellowish-brown colour. The outer membrane fraction, which was white, was located near the 70% sucrose region. The collected fractions were loaded into centrifuge tubes, topped up with dH_2O and centrifuged in a fixed-angle Ti80 rotor at 225,000 x g for 1 h at 4°C. The pellet was then air-dried and resuspended in a Tris-HCl (pH 6.8), 0.001 M dithiothreitol buffer. Samples were stored at -20°C.

b) Bradford determination of protein concentration

The protein concentrations of the inner and outer membrane fractions were determined using the method of Bradford (1976). Dilutions of each membrane fraction were prepared and dH_2O was added to a final sample volume of 0.8 ml. The sample was then mixed with 0.2 ml of Bradford reagent (Biorad, Mississauga, Ontario) and incubated at room temperature for 5 min to allow colour development to occur. The optical density (OD) of each sample was meausred in a spectrophotometer at a wavelength of 595 nm. A standard curve was plotted using bovine serum albumin (BSA; Sigma) in the 1-25 µg range. The protein concentration of each sample was extrapolated from the BSA standard curve.

c) Sodium dodecyl sulfate polyacrylamide gel electrophoresis

Proteins were analyzed using sodium dodecyl sulphate polyacrylamide electrophoresis (SDS-PAGE) with 4% (w/v) stacking and 7.5% (w/v) separating gels (Laemelli, 1970). The gels were polymerized by the addition of 0.1% ammonium persulfate and TEMED to 0.01%. The samples were solubilized at 100°C for five minutes in an equal volume of 2X sample buffer. A high molecular weight standard was also boiled and loaded onto the gel. A discontinuous buffer system was used (0.192 M glycine, 0.02 M Tris-HCl [pH 8.4], 0.1% SDS).

The gels were run at 100V until the samples entered the separating gel, when the voltage was increased to 150V. The samples were run until the dye front ran off the bottom of the gel. The stacking gel was removed and the separating gel was either stained with Coomassie Brilliant Blue or electrophoretically transferred to nitrocellulose for Western immunoblotting. Gels were stained in Coomassie Brilliant Blue R250 (0.05% in 40% methanol, 10% acetic acid) (Eastman Kodak) overnight and then destained in a methanol: acetic acid solution.

d) Western immunoblot analysis

The proteins on the acrylamide gel were transferred to a nitrocellulose membrane according to the method of Burnette (1981). The gel was soaked in blotting buffer (0.192 M

glycine, 0.025 M Tris-Cl [pH 8.4], 20% methanol) for 10 minutes to remove the SDS. A piece of nitrocellulose membrane (Schleicher and Shuell, Willowdale, Ontario) cut to fit the gel was also soaked in blotting buffer. The proteins were transferred to the nitrocellulose membrane in a Bio-Rad Transblot apparatus at 450 mA for 3 h. A water-cooling system was used to prevent heating and breakdown of the blotting buffer.

After electrophorectic transfer, the nitrocellulose membrane was soaked in 3% gelatin in TTBS buffer (0.02 M Tris-Cl [pH 7.5], 0.5 M NaCl, 0.05% Tween-20) for 30 min to block the membrane. The nitrocellulose membrane was transferred to a 1/500 dilution of the first antibody in 1% gelatin and incubated overnight at room temperature with gentle shaking. The membrane was then washed twice in TTBS buffer (15 min per wash) and placed in the second antibody solution (1/2000 dilution) for an hour. The second antibody was goat anti-rabbit or goat anti-bovine IgG-alkaline phosphatase conjugate (Bio-Rad) in 1% gelatin. The membrane was washed twice in TTBS buffer (15 min per wash) and then twice (5 min per wash) in NBT buffer (0.1 M Tris-Cl [pH 9.5], 0.1 M NaCl, 50 mM $MgCl_2$). The membrane was then placed in the developing solution of 100 µl of each of the reagents 5-bromo-4-chloro-3-indolyl phosphate (BCIP, 25 mg/ml in dimethylformamide; Sigma) and nitro-blue-tetrazolium (NBT, 50 mg/ml in 70% dimethylformamide; Sigma). Colour development was allowed to proceed until the desired visibility of the bands was obtained. The colour reaction was stopped by rinsing the membrane in H_2O. The membrane was air-dried.

e) T7 protein expression

Proteins encoded by a recombinant plasmid were analyzed using the method of Tabor and Richardson (1985). The *tbp*A gene was cloned into the plasmid vector pBluescipt. The recombinant plasmid was transformed into *E. coli*JM109 (DE3), which is a strain of *E. coli* JM109 with the T7 RNA polymerase gene integrated into the chromosome and T7 polymerase gene under the control of the lac promoter (Yaninsch-Perron *et al.*, 1985).

After transformation, the cells were grown overnight at 37°C in Davis minimal medium containing 1.0% casamino acids, 0.4% glucose and the appropriate antibiotics. A 1/50 subculture into 20 ml of the same medium was prepared and incubated at 37°C for an additional 3-4 h until the OD_{550}=0.6. The cells were collected by centrifugation at 14,000 x g for 5 min and the pellet was resuspended in Davis minimal medium with 0.4% glucose. The sample was incubated for 90 min at 37°C, then 100 µl of 5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) were added. After the cells were incubated at 37°C for 20 min, rifampicin was added (final concentration of 400 µg/ml). The sample was incubated at 37°C for 30 min and then labelled for 60 min with 5 µCi[^{35}S]-methionine ("Trans-Label", ICN Biomedical, Quebec). The cells were washed twice in ice-cold PBS and collected by centrifugation at 14,000 x g for 5 min in a microfuge. The pellet was resuspended in 2X SDS-PAGE sample buffer.

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The proteins were separated using SDS-PAGE and the gel was stained with Coomassie blue R250. The gel was then soaked in Amplify™ (Amersham, Oakville, Ontario) for 30 min and dried under vacuum. Autoradiographs were exposed 18-48 h.

RESULTS

I. Preliminary Cloning of Putative*tbpA, tbpB* genes

The first stage in cloning the *tbpA* gene was to screen a *P. haemolytica* A1 gene library by polymerase chain reaction. An oligo primer specific for the N-terminal amino acid sequence of the Tbpl protein was synthesized. A *P. haemolytica* A1 codon table (Lo, 1992) was used to optimize the primer sequence. The Tbpl primer was used in conjuction with primers based on the junction sequences of the cloning vector pBR322 (Table 1). A 0.8 kbp PCR product was obtained (Figure 1) and cloned into the M13 vector and sequenced. Sequence analysis of this PCR product demonstrated that the first twenty amino acids matched the sequence obtained by N-terminal amino acid sequencing of Tbpl.

Table 1. Oligonucleotide primers used in PCR.

Primer	Primer Sequence	Size
Tbpl	thr-glu-asn-lys-lys-ile-glu-glu 5'GGAAGCTTACT-GAA-AAT-AAA-AAA-ATC-GAA-GAA *	32 mer
primer left	5' GGAATTCCCGTCCTGTGGATC **	22 mer
primer right	5' GTGAATTCCGGCGTAGAGGATC **	22 mer

* - the underlined sequence is the *Hind*III site
** - the underlined sequence is the *Eco*RI site

The 0.8 kb PCR product was then radiolabelled and used as a specific probe to screen the *E. coli* clones containing *P. haemolytica* A1 gene library by Southern hybridization. Two recombinant clones, 9 and 10, hybridized strongly with the *tbpA* probe. The plasmid DNA from each recombinant clone was analyzed by restriction endonuclease mapping (Figure 2). The insert of *P. haemolytica* A1 was determined to be approximately 8.7 kb and 2.3 kb for plasmids 9 and 10, respectively. Initial sequence analysis of the plasmids confirmed that the insert DNA from both plasmids share an overlapping region. Plasmid 9 contained the entire *tbpA* gene but the region directly uptstream of *tbpA* was different than the upstream region in plasmid 10. It is possible that the insert DNA in plasmid 9 was formed from two DNA fragments from separate regions of the genome. In plasmid 10, the region directly upstream of *tbpA* contained an additional open reading frame which corresponded to the *tbpB* gene. This plasmid, therefore, contained not only the 5' region of *tbpA* but also part of the *tbpB* gene, directly upstream from the *tbpA* gene.

A third recombinant clone, 482, contained the entire *tbpB* gene. This plasmid shares an overlapping region with plasmid 10 (Figure 2). The insert DNA in plasmid 482 is a PCR product obtained from *P. haemolytica* A 1 genomic DNA using primers specific for the amino acid sequence of Tbp2 protein. This PCR product was then cloned into the vector PCRII.

The 3.0 kbp *tbpA* gene was sequenced from plasmid 9 (starting from the *Bgl*II site). The 2.1 kbp *tbpB* gene and the 91 bp sequence between *tbpA* and *tbpB* was sequenced from plasmids 482 and 10.

II. Sequence Analysis

The 5.2 kbp of DNA was sequenced and shown to contain two open reading frames arranged in tandem, with *tbpB* upstream of *tbpA* (Figure 3). or SEQ. ID. NOS. 5 and 6 This genetic organization is consistent with other iron uptake systems in other bacteria where the genes often arranged in an operon (Payne, 1988). Upon sequence analysis of the deduced Tbpl protein, a putative 28 amino acid leader peptide was observed. A putative cleavage sequence for lipoproteins was observed in the deduced Tbp2 protein. The close proximity of *tbpA* and *tbpB* and the absence of a promoter region in *tbpA* suggests that the two proteins may be coordinately expressed. A Fur consensus sequence in the promoter region of *tbpB* suggests that the proteins may be regulated in a Fur-like manner. The Fur consensus sequence in *P. haemolytica tbpB* is similar to the consenses sequence found in *N. gonorrhoeae* and *N. meningitidis tbpB* (Figure 4). or SEQ. ID. NOS. 7, 8, and 9 The isoelectric point of Tbp1 and Tbp2 was calculated by PCGene (Chargpro) to be 9.16 and 9.71 respectively, making them basic proteins.

III. Predicted Protein Topology

The sequence analysis program Gene Runner (Hastings Software) was used to analyze the physical characteristics and to predict the secondary structure of the *P. haemolytica* Tbp1 and Tbp2 proteins. The hydropathy plot of Tbp1 and Tbp2 were generated using the method of Kyte and Doolittle (1982). The first 28 amino acids of Tbp1 form a hydrophobic region, which is characteristic of all signal sequences. There are six other hydrophobic regions in the protein, which may be transmembrane domains of the protein. Hydrophilic areas of the protein may be either exposed at the cell surface or in the periplasm. The hydropathy plot of *N. gonorrhoeae* was also generated. The *N. gonorrhoeae* Tbp1 protein seems to be less hydrophobic than *P. haemolytica* Tbp1 but the location of some of the hydrophobic regions are similar. For example, both proteins have hydrophobic regions around the 200, 400, and 780 amino acid residues. The similarity in hydrophobic regions suggests that the two proteins share a significant degree of homology and may have a similar structure.

The Kyte-Doolittle plot of *P. haemolytica* Tbp2 reveals several large hydrophobic regions in the centre of the protein and two smaller hydrophilic regions at each end. This is significantly different than the hydropathy plot of *N. gonorrhoeae* Tbp suggesting that the two proteins may have different structures.

Surface exposed regions of Tbp1 and Tbp2 were determined using the Emini surface probablility method (Emini *et al.*, 1985). The peaks on the graph correspond to the regions with the highest probability of being exposed. Surface exposed regions of each protein may be involved in ligand binding and may be antigenic. The Emini plot of *P. haemolytica* Tbp1 suggests that the hydrophilic regions near amino acids 330, 410, 460, 560, 610 and 820 of the

protein may be exposed at the cell surface. The Emini plot of *N. gonorrhoeae* Tbp1 showed a few common exposed regions with *P. haemolytica* Tbp1 (regions at 330, 580, 810).

The Emini plot of *P. haemolytica* Tbp2 suggests that the hydrophilic regions at amino acids 140, 160 and 620 have the greatest probability of being exposed. The Emini plot of *N. gonorrhoeae* Tbp2 shows that only the exposed regions at 160, 330 are similar to the surface regions of *P. haemolytica* Tbp2.

The secondary structure of both Tbp1 and Tbp2 were predicted using the method of Chou-Fasman (1978). The Chou-Fasman plot of *P. haemolytica* Tbp1 predicts that the protein is primarily a β-sheet and β-turn structure. The Chou-Fasman plot of *N. gonorrhoeae* Tbp1 predicts a similar structure. These predictions are consistent with other topology predictions of other iron-regulated outer membrane proteins, which are also composed of amphipathic β-sheets (Moeck *et al.*, 1994). It is also interesting to note that the location of the β-sheets correspond to the location of the hydrophobic domains in the two Tbpl Kyte-Doolittle graphs.

The Chou-Fasman plot of *P. haemolytica* Tbp2 indicates that it also consists primarily of β-Sheets and β-turns. The predicted pattern of B-sheets is different than *N. gonorrhoeae* Tbp2, which also has β-sheet regions. These results add to the evidence that the Tbp2 proteins have different structures.

IV. Distribution of *tbpA* in *P. haemolytica* and related species

Southern hybridization analysis was carried out to determine whether or not all sixteen serotypes of *P. haemolytica* carried the gene for the Tbpl protein. Chromosomal DNA from each of the serotypes was disgested with restriction endonuclease and probed with the 5' end of the *typA* gene from *P. haemolytica* A1. Similar hybridization experiments were performed on digested chromosomal DNA from *A. pleuropneumoniae* CM% and shope 4074 and *A. suis* 3714.

High-stringency Southern hybridization (50% formamide) with the *P. haemolytica* A1 *tbpA* probe demonstrated the presence of *tbpA* homologous sequences in all sixteen serotypes of *P. haemolytica* (Figures 5,6). In addition, there is a considerable difference in the size of fragments which hybridized with the probe between the A and T biotypes. It is important to note that in Figure 5, there was a problem with the quality of the serotype 7 DNA, which did not give a reactive band with Southern hybridization. The reaction of this serotype should be identical to that of serotype 1. A similar problem is seen in Figure 6, where serotypes 12 and 16 DNA was not properly digested. The reaction of these serotypes should be identical to that of serotype 1.

Low-stringency Southern hybridization (25% formamide) with the *tbpA* probe indicated that *A. suis* 3714, *A. pleuropneumoniae* CM5 and Shope 4074 genomic DNA hybridized with the *P. haemolytica tbpA* probe (Figure 7). The two strains of *A. pleuropneumoniae* both belong to serotype 1 and hybridized in the same fashion. A preliminary restriction map of the *tbpA, tbpB* regions in *P. haemolytica* A1, *A. suis* and *A. pleuropneumoniae* is shown in Figure 8.

V. Homology Studies

The predicted amino acid sequence of *P. haemolytica* Tbp1 was compared with the predicted sequences for the *Neisseria* spp. and *A. pleuropneumoniae* transferrin binding proteins as well as for several *E. coli* TonB-dependent receptor proteins. All of the comparisons were performed according to the Higgins and Sharp algorithm (Higgins and Sharp, 1988).

The predicted amino acid sequence of *P. haemolytica* Tbp1 was found to have a high degree of homology with both the *N. gonorrhoeae* and *N. meningitidis* Tbp1 proteins (Cornelissen *et al.*, 1992; Legrain *et al.*, 1993) (Figure 9 or SEQ. ID. NOS. 10, 11, and 12). The homology, including identical and conserved amino acids, was found to be 41%. This result agrees with the protein topology studies which suggested that the *P. haemolytica* and *Neisseria* spp. Tbp1 proteins share a similar structure. A homology comparison between *P. haemolytica* Tbp1 and *A. pleuropneumoniae* serotype 7 and serotype 1 TfbA proteins (Gerlach *et al.*, 1992a; Gerlach *et al.*, 1992b) reveals only a low degree (22%) of homology (Figure 10 or SEQ. ID. NOS. 13, 14, and 15). The degree of genetic relatedness among the *Pasteurella, Neisseria,* and *Actinobacillus* transferrin binding proteins is shown in the form of a dendrogram in Figure 11. It is interesting to note that *P. haemolytica* Tbp1 is more closely related to *Neisseria* Tbp1 than to *Actinobacillus* transferrin binding proteins.

P. haemolytica Tbp1 also has localized regions of homology with *E. coli* TonB dependent outer membrance receptors (Figure 12 or SEQ. ID. NOS. 16, 17, 18, 19, and 20). Homology with these proteins implies the *P. haemolytica* Tbp1 is also a TonB dependent receptor protein. The first homologous domain includes the TonB box, which has been implicated in the direct interaction between TonB and the receptor protein (Bell *et al.*, 1990). The significance of the other homologous domains is not known, however, it is possible that they are also involved in TonB interaction.

VI. T7 expression of Tbp1

T7 expression was performed in order to express the protein encoded by *tbpA* (Figure 13). An attempt to express tbpA by maxi-cell analysis of *E. coli* under iron-depleted and iron-repleted conditions was not successful (data not shown). The T7 expression did not produce any reactive band at 100 kDa. A 30 dDa positive control is shown in lane 1. There was no difference between the plasmid carrying *tbpA* and the pBluescript vector alone (Figure 13, lanes 2 and 3).

VII. Western Immunoblot Analysis

Inner and outer membrane fractions from *P. haemolytica* A1 and *E. coli* HB101 cells grown under iron-limiting and iron-sufficient conditions, were prepared and analyzed by Western immunoblotting. The purpose of these experiments was to determine whether or not the iron-regulated proteins would react antigenically with antiserum prepared against the soluble antigens of *P. haemolytica*. The inner and outer membrane fractions were immunoblotted with rabbit "anti-autologous" antiserum which was raised against the soluble antigens of *P. haemolytica* A1 cultured in RPMI 1640 that had been supplemented with the rabbit's own serum (to avoid inclusion of antibodies to serum proteins). The antiserum was preabsorbed with *E. coli* HB101 cells in order to minimize reactivity with *E. coli* antigens. Immunoreactive bands

corresponding to the transferrin binding proteins were not observed in the outer membrane fraction from *P. haemolytica* A1 cells grown under iron-limiting conditions (Figure 14, lane 3).

The inner and outer membrane fractions were also immunoblotted with the serum from a calf vaccinated with Presponse® as a first antibody (Figure 15). The antiserum was preabsorbed with *E. coli* HB101 cells to limit the number of E. coli immunoreactive. Bands of 71, 77 and 100 kDa were observed in the outer membranes of *P. haemolytica* cells which were grown under iron-limiting conditions (Figure 15, lane 3). These protein bands correspond to the size of the *P. haemolytica* transferrin binding proteins. If these antigenic bands are the transferrin-binding proteins then this result suggests that these peptides are antigenic and also immunogenic in cattle.

DISCUSSION

I. Preliminary Sequence analysis

The preliminary nucleotide sequences of *P.haemolytica tbpA* and tbpB are shown in Figure 3. The promoter region of *P. haemolytica tpbB* is shown in Figure 4.

Upon preliminary sequence analysis of the cloned DNA, the two *tbp* genes were found in tandem with *tbpB* directly upstream of *tbpA*. This genetic organization is consistent with iron uptake systems in other bacteria such as *Neisseria* spp. where the genes where are arranged in an operon (Anderson at *al., 1994).* It is likely that the genes involved in *P. haemolytica* A1 iron uptake are also arranged in an operon. The *tbpA* gene has only a ribosomal binding sequence whereas the *tbpB* gene is preceded by a ribosomal binding site and has a Fur consensus sequence in its promoter region.

The presence of a putative Fur consensus sequence implies that the two genes could be coordinately regulated by iron concentrations and that a Fur homolog exists in *P. haemolytica* A1. Fur homologs have been cloned and sequenced in pathogenic *Neisseria* Spp. (Berish *et al.*, 1993; Thomas and Sparling, 1994). If a *P. haemolytica* A1 Fur homolog exists, it may be involved in the regulation of other antigens such as the leukotoxin. Strathdee and Lo (1989) reported that under iron-limiting conditions, there was a decrease in the amount of leukotoxin produced. This is the opposite of the situation in the diptheria toxin (Boyd *et al.*, 1990) where toxin production increases when the cells are grown under iron-limiting conditions. It is possible that Fur acts as a positive regulator in *P. haemolytica* leukotoxin production. This would be consistent with the earlier observation by Gentry *et al.* (1986) of increased toxin production in iron containing media. *N. meningitidis* also produces iron-regulated proteins which are related to the RTX family of exoproteins (Thompson *et al.*, 1993).

The first 28 predicted amino acids in the *tbpA* sequence form a putative signal sequence. A signal sequence is essential for inserting the precursor protein into the membrane during the process of translocation across the membrane. The signal sequence also acts as a recognition site for the proteolytic cleavage of the precursor protein into its mature form (von Heijne, 1983; Benson and Silhavy, 1983). The presence of a signal sequence confirms that Tbp1 is

located beyond the cytoplasmic membrane, but it does not contain any sorting information. The predicted amino acid sequence of Tbp1 has a terminal phenylalanine residue at the carboxyl-terminal of the protein. Phenylelanine is a hydrophobic aromatic amino acid which facilitates the partitioning of the hydrophobic environment in the membrane. The presence of a terminal phenylalanine residue has been shown to be important for outer membrane localization (Struve *et al.*, 1991) and suggests that *P. haemolytica* Tbp1 is located in the outer membrane.

Sequence analysis of *tbpB* revealed a putative cleavage sequence for lipoproteins. Lipoproteins have a characteristic cleavage sequence of Leu-X-Y-Cys, where X and Y are small neutral amino acids (Wu, 1987). This suggests that Tbp2 is processed and lipid modified. It is interesting to note that Tbp2 lacks a terminal phenylalanine residue which is involved in outer membrane localization. Analogous transferrin binding lipoproteins have been found in *H. influenzae, N. gonorrohoeae* and *N. meningitidis* (Legain *et al.*, 1993; Anderson *et al.*, 1994) Griffiths *et al*. (1993) has demonstrated common antigenic domains among the Tbp2 proteins in *N. gonorrohoeae, N. meningitidis* and *H. influenzae* type b.

The isoelectric point (pI) of a protein is defined as the pH at which the peptide has a net charge of zero. The pI calculation assumes that there are no three-dimensional structures which interfere with the ionization states. Therefore, calculated pI values are only approximate values and may differ from experimental results. The pI of *P. haemolytica* Tbp1 and Tbp2 has been calculated to be 9.16 and 9.71, respectively. It has been suggested that cationic polypeptides enhance *in vivo* membrane interactions. It is possible that the basic nature of Tbp1 enhances interaction with the host transferrin proteins. The *Neisserial* Tbp1 (Cornelissen et al., 1992) and Fbp (Berish et al., 1990) proteins are also basic proteins. In *Legionella pneumophilia*, basic surface proteins act to inhibit phagolysosomal fusion (Cianociotto et al., 1989).

II. Preliminary Predicted protein topology

The hydropathy plots of Tbp1 and Tbp2 were generated using the method of Kyte and Doolittle (1982). In the hydropathy plot of *P. haemolytica* Tbp1, the first peak is located from the amino acids 1 to 30. This represents the hydrophobic core which is common to all signal sequences (Hayashi and Wu, 1990). The other hydrophobic regions may be transmembrane domains. Hydrophilic domains may be regions of the protein which are exposed to either the cell surface or to the periplasm. The location of the transmembrane regions in the *P. haemolytica* Tbp1 protein is similar to many transmembrane regions predicted in *N. gonorrhoeae* Tbp1. This suggests that the Tbp1 proteins may have a similar structure and that they share a certain degree of homology at the amino acid level. The *P. haemolytica* Tbp2 protein possesses a hydrophobic leader sequence as well as several large hydrophobic regions in the centre of the protein and it is significantly different than the hydropathy plot predicted for *N. gonorrhoeae* Tbp2. This implies that both proteins are structurally different.

Surface exposed regions of Tbp1 and Tbp2 were predicted using the Emini surface probability method. Regions of Tbp1 and Tbp2 which are exposed to the cell surface may be involved in ligand interaction and may be antigenic.

The Chou-Fasman method is commonly used in predicting the secondary structure of a protein. This method is based on the tendency each amino acid has for being in an *a*-helix, a β-sheet or a *β*-turn. The Chou-Fasman method predicts that *P. haemolytica* Tbp1 consists of many *β*-sheets and *β*-turns. It is possible that these β-sheets cross the outer membrane repeatedly and that the intervening sequences constitute surface or periplasm exposed loops. The Chou-Fasman method also predicts that *N. gonorrhoeae* Tbp1 also consists of β-sheets. This structure has already been proposed for *E. coli* outer membrane proteins such as FepA (Moeck *et al.*, 1994). *P. haemolytica* and *N. gonorrhoeae* Tbp1 proteins share a common structure which implies that they may also have a similar mechanism of removing iron from the host transferrin molecule. The Chou-Fasman plot of *P. haemolytica* Tbp2 also predicts a predominantly *β*-sheet and *β*-turn structure which is significantly different than the prediction for *N. gonorrhoeae* Tbp2.

III. Distribution of *tbp*A in *P. haemolytica* and Related Species

Southern hybridization of the genomic DNA of the sixteen *P. haemolytica* serotypes with the *tbpA* probe demonstrated that a highly homologous gene is present within the A biotype. The results also suggest that the genetic organization or the *tbpA* gene is significantly different in the T biotypes. This supports the observations by Murray *et al.* (1992) who demonstrated that iron-regulated proteins from the A and T biotypes were antigenically distinct. Previous work on *P. haemolytica* antigenic determinants demonstrated that the sialoglycoprotease, the serotype specific antigen, three lipoproteins and a LPS biosynthetic gene were either missing or had a different genetic organization in the T biotype (Burrows, PhD thesis, 1993). The A and T biotypes do share phenotypic and biochemical traits (Holt, 1977), but they are only distantly related based on DNA:DNA hybridization (Bingham et al., 1990). Sneath and Stevens (1990) proposed that the biotype T serotypes be renamed as the species *P. trehalosi*.

Diversity in the genetic organization of transferrin binding proteins has also been demonstrated in *A. pleuropneumoniae* TfbA (Gonzalez *et al.*, 1990; Gerlach *et al.*, 1992b) and *N. meningitidis* Tbp2 (Legrain *et al.*, 1993; Rokbi, 1993). In *A. pleuropnuemoniae*, serotype 1 and serotype 7 TfbA proteins share only 55% homology at the amino acid level (Gerlach *et al.*, 1992b). *N. meningitidis* Tbp2 proteins are divided into two classes based on their molecular weight, sequence similarity and antigenic heterogeneity (Robki *et al.*, 1993). The diversity in transferrin binding proteins within a species may facilitate the binding of different serotypes to avoid the host immune response against heterologous strains (Gerlach *et al.*, 1992b).

Southern hybridization experiments demonstrated that chromosomal DNA from *A. pleuropneumoniae* strains CM5 and Shope 4074 hybridized with the *tbp*A probe only under low-stringency conditions. This suggests that *P. haemolytica* and *A. pleuropneumoniae* transferrin

binding proteins share only a low degree of homology. This result was confirmed by homology studies on the amino acid sequence from both *P. haemolytica* Tbp1 and *A. pleuropneumoniae* TfbA proteins.

The *A. suis* genomic DNA also hybridized with the *tbp*A probe, which suggests that it may have an analogous transferrin binding protein. The results also suggest that *A. suis* transferrin binding proteins may be more closely related to the Tbp1 and *P. haemolytica* than to *A. pleuropneumoniae* TfbA.

IV. Homology Studies

All of the protein sequences were aligned by PCGene (Clustal), which compares sequences according to the method of Higgins and Sharp (1988). The first step in this method is to calculate all pairwise sequences similarities. A dendrogram is then generated from the similarity matrix generated in the first step. The dendrogram in Figure 11 was generated by a Higgins and Sharp alignment of the *Pasteurella, Actinobacillus*, and *Neisseria* transferrin binding proteins.

***a) Neisseria* spp.**

The predicted amino acid sequence of *P. haemolytica tbpA* has regions of homology with the predicted amino acid sequence of *tbpA* in *N. gonorrhoeae*. This suggests that the transferrin binding proteins are structurally similar and agrees with the observations made in the protein topology studies. Ogunnariwo and Schryvers (1990) reported that *P. haemolytica* A1 Tbp1 was similar to *N. gonorrhoeae* Tbp1 proteins in size and properties. Both species produce 100 kDa receptor proteins which cannot bind transferrin after SDS-PAGE, which suggests that the conformation of the native protein is important in transferrin binding. However, the two proteins differ in their binding specificities: *N. gonorrhoeae* Tbp1 bound only human transferrin whereas *P. haemolytica* A1 Tbp1 bound only bovine transferrin. This suggests that differences between the two *tbpA* sequences may be regions which encode for specificity of iron source.

b) *A. pleuropnuemoniae*

The predicted amino acid sequence of *P. haemolytica* A1 Tbp1 has a low degree of homology with the sequence of *A. pleuropneumoniae* TfbA. This result is confirmed by the Southern hybridization experiments, which demonstrated that chromosomal DNA from *A. pleuropneumoniae* strains CM% and Shope 4074 hybridized with the *tbp*A probe only under low-stringency conditions. This is interesting because both bacteria belong to the family *Pasteurellaceae* and would therefore be expected to have a similar transferrin binding protein. Previous work has suggested that the two proteins are functionally similar but structurally different. The TfbA protein in *A. pleuropneumoniae* has been shown to be a lipoprotein (Gonzalez *et al.*, 1990), whereas the 100 kDa *P. haemolytica* A1 Tbp1 is not (Ogunnariwo and Schryvers, 1990). *A. pleuropneumoniae* is able to distinguish between iron-saturated and iron-

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depleted transferrin) (Gerlach *et al.*, 1992a), whereas *N. meningitidis* cannot (Tsai *et al.*, 1988). It is interesting to note that *A. pleuropneumoniae* TfbA has homology with *N. gonorrhoeae* Tbp2, which is also a lipoprotein. This suggests that the TfbA protein is analogous to Tbp2, and that Tbp1 of *A. pleuropneumoniae* has not yet been identified.

c) TonB dependent-receptor proteins

The *P. haemolytica* Tbp1 sequence also has amino acids which are common to a group of *E. coli* TonB dependent receptor proteins. This finding suggests that *P. haemolytica* A1 belongs to this family and that a TonB homolog exists in *Pasteurella* species. The first homologous domain or "TonB box" has been implicated in direct interaction between the receptor protein and TonB (Bell *et al.*, 1990, Brewer et al., 1990). The significance of the other homologous regions is not known, but they may be required for TonB interaction or may be necessary for outer membrane localization. *P. haemolytica* Tbp1, like many other TonB-dependent proteins, is a transmembrane protein which is iron-regulated and involved in iron utilization (Mietzner and Morse, 1994). It is possible that *P. haemolytica* Tbp1 functions as a gated channel as has been proposed for *E. coli* FepA (Rutz *et al.*, 1992). Tbp1 from both *N. gonorrhoeae* (Cornelissen *et al.*, 1992), and *H. influenzae* (Jarosik *et al.*, 1994) also belong to the family of TonB-dependent receptor proteins.

V. Proposed Model for *P. haemolytica* Iron Uptake

The existence of many analogous proteins in *Neisseria, Pasteurella* and *Haemophilus* suggests that a common mechanism may be utilized for iron acquisition. A hypothetical model of iron acquisition has been proposed for *Neisseria* (Chen *et al.*, 1993) which may be used as a model for *P. haemolytica* A1. Iron deprivation activates transcription of the iron-regulated proteins by a Fur-like regulatory system. Host transferrin binds to the bacterial cell surface via a specific iron receptor complex composed of two or more proteins. The iron is removed from the transferrin and transported across the outer membrane of the bacterium with the energy provided by TonB. In the periplasm, the iron is transiently complexed to a periplasmic component, Fbp, which transports it to a cytoplasmic membrane permease. The iron is transported across the cytoplasmic membrane by a periplasmic binding protein transport system. In the cytoplasmic the iron is reduced to Fe^{2+} and assimilated by the cell.

One feature which may be unique to *P.haemolytica* A1 iron uptake is the presence of a third iron-regulated outer membrane protein (71 kDa) which may form part of the receptor complex (Ogunnariwo and Schryvers, 1990). In addition, *P. hemolytica* does not have a receptor protein which is capable of binding transferrin after SDS-PAGE and electroblotting, while *N. gonorrhoea* does (Schryvers and Morris, 1988). This suggests that the binding mechanism of *P. haemolytica* receptor complex may be slightly different than the receptor complex in *N. gonorrhoea.*

Proteins which are similar to *N. gonorrhoea* Fbp have been identified in the family Pasteurellacease. In *H. influenzae,* a 40 kDa periplasmic protein was identified and its N-

terminal sequence was found to be 81% homologous to *N. gonorrhoea* Fbp (Harkness et al., 1992). In *P. haemolytica* A3, 35 kDa periplasmic iron-regulated protein has been described but no function has been found (Lainson et al., 1990). In addition, a 37 kDa iron regulated protein has been isolated by affinity procedure from *P. haemolytica* A1 (Ogunnariwo and Schryvers, 1990). Based on size and location similarities, it is possible that both of these proteins are analogous to *N. gonorrhoea* Fbp.

VI. T7 Protein Expression

T7 RNA polymerase-dependent production of a Tbp1 gene product in *E.coli* JM109 (DE3) was not successful (Figure 13). One possible explanation may be that the ribosomal binding site of *tbpA* was inefficient. The gene could perhaps be cloned into a vector that carries a functional ribosome-binding site. Alternatively, the Tbp1 protein may be unstable and requires the presence of other proteins or factors in order to be correctly produced. Components of heterodimeric proteins are often unstable when they are synthesized singly.

VII. Western Immunoblot Analysis

Western immunoblots were performed on the inner and outer membrane fractions from *P. haemolytica* A1 cells which were grown under iron-sufficient or iron-limiting conditions (Figures 14 and 15). The iron-limiting conditions were simulated by adding the iron chelator EDDA, which is a common synthetic iron chelator used to limit the availability of iron in culture media. EDDA was chosen for these studies because of its specificity for iron and its lack of toxic side effects to bacteria (Neilands, 1981).

P. haemolytica A1 membrane fractions immunostained with rabbit antiserum to soluble antigens did not react with the iron-regulated proteins (Figure 14). Neither the 100 kDa nor the 77 kDa iron-regulated proteins were observe in this immunoblot, possibly because the original *P. haemolytica* A1 culture (used in the hyperimmunization of the rabbit) was not grown under iron-restricted conditions. The medium used contained 7% serum to avoid the inclusion of antibodies of serum proteins. In contrast, peptides which may be the iron-regulated proteins reacted with antisea from calves vaccinated with the Presponse® (Figure 15). Presponse® is produced from *P. haemolytica* A 1 cells which are grown to late log phase in serum-free RPMI medium 1640 (Shewen and Wilkie, 1987; Shewen et al., 1988). It is possible that the low iron concentration of this medium induced production of the transferrin binding proteins. It is also possible that the calf responded to transferrin binding proteins produced by *P. haemolytica* which are commensal organisms in the nasopharynx. The presence of antibodies to transferrin binding proteins suggests that these proteins are immunogenic.

EXAMPLE 2

Bacterial strains. The bacterial strains used in this study are listed in Table 2. *P. haemolytica* strains h173, h174, h175 and h176 were field isolates from ruminants with pneumonic pasteurellosis and were provided by Dr. Frank Milward, Rhone Merieux, Lyon, France. *P. haemolytica* strains h44-h46 were bovine clinical type A1 isolates from bovine pneumonia

obtained from S. Lundberg ,Veterinary Laboratory, Regional Agricultural Building, Airdrie, Alberta. h44 has been described previously (26). *P. haemolytica* strains h93-h97 were bovine clinical type A1 isolates from bovine pneumonia obtained from by Dr. A. Potter of the Veterinary and Infectious Diseases Organization (VIDO), Saskatoon. Strains h98-h107 are ATCC *P. haemolytica* strains (5) also obtained from Dr. A. Potter. *Actinobacillus (Haemophilus) equuli* strain h50 was obtained from Dr. Jane Pritchard, Veterinary Laboratory, Regional Agricultural Building, Airdrie, Alberta. new species, *P. trehalosi* (34).

Table 2. List of strains included in this study.

Species	Strain	Serotype	Host Species	Source
P. haemolytica	h44	A1	cattle	S. Lunberg, Airdrie
P. haemolytica	h45	A1	cattle	S. Lunberg, Airdrie
P. haemolytica	h46	A1	cattle	S. Lunberg, Airdrie
P. haemolytica	h93 (ph21)	A1	cattle	A. Potter, VIDO
P. haemolytica	h94 (ph24)	A1	cattle	A. Potter, VIDO
P. haemolytica	h95 (ph27)	A1	cattle	A. Potter, VIDO
P. haemolytica	h96 (ph45)	A1	cattle	A. Potter, VIDO
P. haemolytica	h97 (ph46)	A1	cattle	A. Potter, VIDO
P. haemolytica	h196	A1	cattle	R. Lo, U. of Guelph
P. haemolytica	h98 (ATCC33366)	A2	sheep	A. Potter, VIDO
*P. haemolytica**	h99 (ATCC33367)	T3	sheep	A. Potter, VIDO
*P. haemolytica**	h100 (ATCC33368)	T4	sheep	A. Potter, VIDO
P. haemolytica	h101 (ATCC33370)	A6	sheep	A. Potter, VIDO
P. haemolytica	h102 (ATCC33371)	A7	sheep	A. Potter, VIDO
P. haemolytica	h103 (ATCC33372)	A8	sheep	A. Potter, VIDO
P. haemolytica	h104 (ATCC33373)	A9	sheep	A. Potter, VIDO
P. haemolytica	h105 (ATCC33369)	A5	sheep	A. Potter, VIDO
*P. haemolytica**	h106 (ATCC33374)	T10	sheep	A. Potter, VIDO
P. haemolytica	h107 (ATCC33375)	A11	goat	A. Potter, VIDO
P. haemolytica	h173 (77020-15184)	Untypable	goat	F. Milward, Rhone Merieux
P. haemolytica	h174 (90020-16266)	A7	goat	F. Milward, Rhone Merieux
P. haemolytica	h175 (84020-15786)	A7	sheep	F. Milward, Rhone Merieux
P. haemolytica	h176 (84020-15792)	A9	sheep	F. Milward, Rhone Merieux
A. equuli	h50		horse	J. Pritchard, Airdrie

* T-type strains are now considered as a new species, *P.trehalosi* (34).

Growth conditions. All bacterial strains were stored frozen at -70°C in 30% glycerol. Isolates from the frozen stocks were streaked onto chocolate agar plates and incubated at 37°C in a 5% CO_2 incubator. Iron-restricted growth was achieved by growing the bacteria in Brain Heart Infusion broth (BH1, Difco Laboratories) or O'Reilly Niven broth (25) supplemented with 2 μg/ml thiamine monophosphate and 3 μg/ml nicotinamide adenine dinucleotide (NAD) and containing the iron chelator ethylenediaminedihydroxyphenylacetic acid (EDDHA, Sigma) at a final concentration of 100 μM. Growth experiments for use of different transferrins as iron source was performed as previously described (26).

Preparation of transferrins and derivatives. Bovine transferrin was obtained from Sigma. The preparation of equine (horse), ovine (sheep) and caprine (goat) transferrins (2), the iron loading of transferrins to 30% or 100% saturation (22) and conjugation of horse-radish peroxidase (HRP) to trannsferrin (37) was essentially as described previously. In the preparation of conjugates of bovine, ovine, caprine and equine transferrins (HRP-bTf, HRP-oTf, HRP-cTf and HRP-eTf), the mixture of HRP and transferrin were subjected to gel filtration after chemical conjugation. The fractions demonstrating maximal activity were pooled, dialyzed and aliquots frozen and stored at -70°C.

Solid-phase binding assays. The solid phase binding assay was essentially derived from methods described previously (32). Aliquots of intact cell suspensions or crude total membrane preparations were spotted onto nitrocellulose/cellulose acetate membranes (HA paper, Millipore Corporation, Bedford, MA) and after drying the HA paper was blocked with buffer containing 0.5% skim milk (blocking solution). For the transferrin binding assay, the paper was exposed to blocking solution containing 450 ng/ml of the HRP-conjugated transferrin, washed and developed with HRP substrate mixture essentially as previously described (32). For assessment of binding of anti-receptor antibody by intact cells a similar procedure was utilized except that the first binding solution contained a 1/1,000 dilution of the anti-TbpA and anti-TbpB antisera and, after washing, the membrane was exposed to a second binding solution containing a 1/3,000 dilution of a HRP-conjugated goat anti-rabbit antibody preparation.

Affinity isolation of transferrin binding proteins (TbpA and TbpB). Bovine, ovine, caprine and equine transferrins were individually coupled to CNBr-activated Sepharose 4B according to the manufacturers instructions using solutions containing 3.5 mg/ml of iron-saturated transferrin. Activated groups were blocked by addition of ethanolamine. Noncoupled transferrin was removed by washing with 10 to 20 column volumes of a 50 mM TrisHCl, 1 M NaCl, pH 8.0 buffer containing 6.0 M guanidine hydrochloride and after further washing the bound transferrin was reloaded with iron using a solution containing 5 μg/ml $FeCl_3$ in 0.1 M sodium citrate/0.1 M $NaHCO_3$ pH 8.6 buffer.

Iron-deficient total membrane (200 mg protein) from *P. haemolytica* or *A. equuli* prepared as previously described (32) was diluted to 2 mg/ml in 50 mM Tris pH 8.0 containing 1.0

M NaCl. The diluted membrane was solubilized by addition of EDTA and sarkosyl to a final concentration of 10 mM and 0.75%, respectively followed by incubation of the mixture at room temperature for 15-30 min with gentle rocking. The solution was centrifuged at 10,000 rpm for 10 min to remove insoluble debris. The supernatant containing the solubilized membrane was applied to a 1.5 x 10 cm transferrin-affinity column and then washed extensively (at least 10 bed volumes) with 50 mM Tris pH 8.0 containing 1.0 M NaCl, 10 mM EDTA, 0.75% Sarksosyl to remove non-specifically bound protein. In experiments using low salt washing conditions the washing buffer contained 100 mM NaCl in lieu of 1M NaCl. In some instances, additional washing with 2-3 bed volumes of washing buffer containing 0.2 M guanidine hydrochloride was necessary to remove contaminating proteins.

Coelution of both transferrin binding proteins (TbpA and TbpB) was achieved by application of 2-3 bed volumes of 2.0 M guanidine hydrochloride in 50 mM Tris pH 8.0, containing 1.0 M NaCl, 1 mM EDTA, 0.01% sarkosyl. The eluant was collected for immediate dialysis against 50 mM Tris pH 8.0. Further treatment with higher concentrations of guanidine hydrochloride usually did not result in any further yield of receptor protein. Individual isolation of TbpA and TbpB was attained by sequential elution with 2 bed-volumes of each buffer containing 0.2, 0.5, 0.75, 1.0, 1.5, 2.0 and 3.0 guanidine hydrochloride, respectively. The eluates were dialyzed against 3 changes of 3 litres 50 mM Tris pH 8.0 over an 18-hour period and concentrated by ultrafiltration. After SDS-PAGE analysis the fractions from the 0.5 and 0.75 M guanidine HCl elution buffers were pooled for a preparation of TbpB and fractions from the 1.5 and 2 M guanidine HCl elution buffers were pooled for a preparation of TbpA.

Analytical methods. Protein samples were analyzed by SDS-PAGE followed by silver staining as previously described (32). For Western blot analysis, about 1-2 .m of purified receptor proteins or 40 .m of outermembrane protein from iron-poor cells were separated on 10% polyacrylamide gels. Proteins were electrophoretically transferred to nitrocellulose (Millipore, Bedford, MA) overnight at 15V in 20 mM Tris, pH 7.5, 150 mM glycine, 20% methanol and 0.1% SDS. The filters were blocked with 0.5% skim milk in 20 mM Tris pH 7.5, 500 mM NaCl (TBS) for 30 minutes at room temperature. A 1/300 dilution of the appropriate antibody in the blocking solution was applied to the paper for 1 hour at room temperature followed by two, 10-minute washes each with TBS. A 1/3000 dilution of secondary antibody (goat anti-rabbit IgG-horse-radish peroxidase conjugate from BioRad) was allowed to bind for 1 hour at room temperature. The conjugate was removed by three, 10-minute washes in TBS and developed using the HRP-substrate mixture.

Comparison of receptor specificity:

Prior studies had demonstrated differences in specificities towards different ruminant transferrins (i.e. cattle, sheep and goat) by transferrin receptors from various pathogenic bacterial species of ruminants (38). This probably reflects differences in the regions of the receptor proteins involved in ligand binding and thus suggests that these regions could not

serve as the basis of a broad-spectrum transferrin receptor-based vaccine for ruminant pathogens. However, it does not preclude the possibility that a group of related ruminant pathogens, such as the various *Pasteurella* species, may have common ligand binding domains that could provide the basis for generation of a cross-protective response. Thus it was important to determine whether the transferrin receptors from a collection of representative *Pasteurella* isolates possessed the same specificity for ruminant pathogens.

As a preliminary analysis of receptor specificity, a collection of representative isolates were assessed for their ability to utilize various ruminant transferrins as a source of iron for growth (Table 2). A simple plate assay described in the methods section was utilized. The growth of all the representative ruminant isolates of *Pasteurella haemolytica* and *P. trehalosi* was stimulated by Fe-saturated transferrins from ruminant (bovine, caprine and ovine) but not from non-ruminant (equine) hosts. The stimulation of the growth of the equine pathogen, *Actinobacillus equuli* (strain h50), by equine transferrin indicated that the inability of the *P. haemolytica* strains to use equine transferrin as iron source was not due to deficiencies in the preparation.

Table 3: Growth on different transferrins.

Species	Strain	Serotype	Host	Source of Tf for growth			
				bTf	oTf	cTf	eTf
P. haemolytica	h44	A1	cattle	+	+	+	-
P. haemolytica	h173	Untypable	goat	+	+	+	-
P. haemolytica	h174	A7	goat	+	+	+	-
P. haemolytica	h175	A7	sheep	+	+	+	-
P. haemolytica	h176	A9	sheep	+	+	+	-
P. haemolytica	h106	T10	sheep	+	+	+	-
A. equuli	h50		horse	-	-	-	+

As a further assessment of the receptor specificity, binding of transferrin by intact cells or isolated membranes was assessed by a simple binding assay utilizing horse-radish peroxidase (HRP) conjugates of transferrin. Conjugates were prepared from bovine, ovine and caprine transferrin and then tested for their ability to bind to total membranes isolated from iron-deficient cells of several representative strains of *P. haemolytica* and *P. trehalosi*. The results illustrate that all the selected strains were capable of binding the three ruminant transferrins (bovine, caprine and ovine) but not equine transferrin (Figure 16), which is consistent with the results of the growth studies (Table 3). To confirm that the observed binding by all three ruminant transferrins was due to the same receptor in the selected species, competitive binding assays were performed in which the ability of unlabelled ruminant transferrins were tested for their ability to block binding of the labelled transferrins. In these experiments

reciprocal inhibition by the various ruminant transferrins was equally effective, indicating that they bound to the same receptor with similar affinities (data not shown).

The results of the growth and binding studies suggested that bovine, ovine and caprine transferrins were capable of interacting with the receptor components involved in iron acquisition in *P. haemolytica*. The affinity procedures described in the methods section were used to identify the proteins interacting with the ruminant transferrins by employing bovine, caprine or ovine transferrin-sepharose resins. As illustrated in Figure 17, a predominant receptor protein of approximately 100,000 molecular weight was isolated with membrane preparations from the bovine isolate (h44), the caprine isolate (h173) or the ovine isolate (h175) when either bovine (lanes A and B), ovine (lane C) or caprine (lane D) transferrin affinity columns were used. This protein is analogous to receptor proteins of similar size that are found in other bacterial pathogens (18,27,30,31), which have conventionally been termed transferrin binding protein 1 (Tbp1). An alternate name, TbpA has been recommended (21)to be consistent with existing conventions of nomenclature.

A second protein of approximately 60,000 molecular weight was also evident in the samples isolated by affinity chromatography with the ruminant transferrins (lanes B, C and D) using membranes from the bovine isolate (h44). This protein is comparable to the lower molecular weight receptor protein, transferrin binding protein 2 (Tbp2), isolated from other pathogenic bacterial species (18,27,30,31). For reasons alluded to above, the alternate name, TbpB, has been recommended (21). A protein of this molecular weight is also detectable in most samples obtained with the caprine (h173) and ovine (h175) isolates but the presence and yield of this component was sensitive to the conditions of isolation. The characteristically low yield of TbpB (Tbp2) relative to TbpA (Tbp1) observed in these species is not a general property of the bacterial receptor proteins and may even reflect common properties of TbpB from related species.

Neither of the proteins were isolated when equine transferrin-Sepharose was used in the affinity isolation procedure (lane E) indicating that their isolation was specifically due to the presence of ruminant transferrin. When less stringent washing conditions were used during the affinity isolation procedure, additional proteins of approximately 38,000 and 70,000 molecular weight were retained by the affinity column (lane A) when membranes from the bovine (h44), caprine (h173) or ovine (h175) isolate were used. An additional protein of approximately 77,000 molecular weight was also evident in the sample obtained with membranes from the bovine isolate.

Comparison of the immunological properties of the receptor proteins.

The observation that bovine, caprine and ovine transferrins compete for the same receptors suggested that there is conservation at least in the binding domain of the receptors. In order to determine whether there was also a similarity with respect to presence of common immunological epitopes, antisera were prepared against purified receptor proteins from one strain to evaluate their crossreactivity with receptor proteins from other isolates. Affinity

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purified preparations of TbpA and TbpB were obtained from strain h44 (see methods section) and used for generation of monospecific antisera in rabbits. These antisera were then tested against receptor proteins isolated from representative strains of different serotypes including isolates obtained from cattle, sheep and goats. The results in Figure 18, Panel A demonstrate that the anti-TbpB antisera reacted strongly with a protein of approximately 60,000 molecular weight (TbpB) that was affinity isolated with bTf-Sepharose from all of the representative strains. Similarly, the anti-TbpA antisera crossreacted with TbpA isolated from all seven representative strains (Figure 18, Panel B). Extension of this analysis to the additional serotypes of ruminant isolates (Table 2) continued to show considerable cross-reactivity with both receptor proteins (data not shown). These data suggest that both receptor proteins are conserved amongst the different serotypes of *P. haemolytica* causing pneumonic pasteurellosis in cattle, sheep and goats.

Although the immunological cross-reactivity illustrated in Figure 18 indicates that there are conserved epitopes in receptor proteins from different species, there is no indication whether any of these epitopes are exposed at the bacterial surface, where they could serve as effective targets for the host immune effector mechanisms. In order to address this issue, a solid-phase binding assay was used to assess the binding of antireceptor antibodies by intact cells. This assay demonstrated that there was strong binding by cells grown under iron-deficient, but not iron-sufficient conditions, when a selection of bovine type A1 isolates were tested (data not shown). When a selection of sheep isolates of varying serotypes were tested, there was a variable degree of reactivity (Figure 19). Other serotypes of type A *P.haemolytica* strains (h98 and h105, Figure 19) showed considerable reactivity against the anti-TbpA and anti-TbpB antisera. In contrast, the T-type strains (*P. trehalosi*, h99, h100 and h106) showed only very weak reactivity against both of the anti-receptor antisera. However, the fact that there was also weak binding by labelled bTf indicates that there was limited production of receptor proteins under the iron-deficient growth conditions used in this experiment. Thus the lack of reactivity of the anti-receptor antisera cannot be attributed to a lack of surface-exposed, cross-reactive epitopes in the receptor proteins from these species.

EXAMPLE 3

Cloning of the transferrin receptor genes from a type A1 strain.

The following materials and methods were used in the studies described in the example:

MATERIALS AND METHODS

Bacterial, plasmids, phages and culture conditions. *P.haemolytica* and *E.coli* strains were from the inventors' laboratory collections. The plasmid clone bank of *P. haemolytica* A1 DNA in pBR322 has been described (Lo et al., 1985). The l clone bank containing *P. haemolytica* A1 DNA was obtained from G.Weinstock. *P. haemolytica* A1 strain H196 was from the Veterinary Infectious Diseases Organization (VIDO, Saskatoon, Saskatchewan, Canada). All bacterial

strains were stored frozen at -70°C in 30% glycerol. Isolates from the frozen stocks were streaked onto chocolate (*P. haemolytica*) or Luria-Bertani plus antibiotic (*E. coli*) agar plates and incubated at 37°C in a 5% CO_2 incubator.

PCR amplification. The primers for PCR were synthesized in an Applied Biosystems Model 390E Synthesizer and purified according to manufacturer's instructions. PCR was carried out in thin-walled 500 ml tube in a Perkin-Elmer Cetus 480 Thermal Cycler using the PCR coreagents and Taq DNA polymerase as recommended. The PCR conditions consist of 95° C for 2 min., followed by 30 cycles of denaturation, annealing and extension at 95° C (1 min.), 52° C (1 min.) and 72° C (2min.) respectively. A negative control which did not contain template DNA was included in each PCR run.

Mapping of tbp region in the genome. Genomic DNA from *P. haemolytica* A1 were digested with a number of restriction enzymes, separated by agarose gel electrophoresis, blotted onto nitrocellulose membrane and hybridized with DNA probes specific for the different regions of *tbpA* or *tbpB* as described. The restriction maps were compared with that obtained from the recombinant plasmids as well as the sequenced regions to verify the correct positions of the *tbp* genes.

Preparation of transferrins and derivatives. Bovine transferrin (bTf) was obtained from Sigma. The apo-form of bTf was produced following a procedure described elsewhere (Mazurier and Spik, 1980). Briefly, bTf was dissolved to a concentration of 0.5-1.0 % in 0.1 M Na acetate, 0.1 M Na phosphate, 25 mM EDTA, and adjusted to (pH 5.5) by adding drops of concentrated glacial acetic acid. The solution was equilibrated overnight at 4°C, and the iron removed using an acrylamide gel column equilibrated with the Na acetate/Na phosphate low pH buffer. The low pH buffer was exchanged using a acrylamide gel column equilibrated with 50 mM Tris-HCl (pH 7.5). Finally, the protein was concentrated using Amicon filter. The N-and C-terminal derivatives of bTf were produced as described (Yu and Schryvers, 1994). Briefly, 80 mg of ConA purified bTf were digested with 2 mg proteinase K in 40 ml 0.1 M Tris-HCl (pH 8.2), 25 mM $CaCl_2$ at room temperature for 20h. To stop the reaction, phenylmethylsulfonyl fluoride (PMSF) was added to 0.1 mg/ml. Five ml of concentrated preparation were applied to a Sephadex G-100 column equilibrated with 50 mM Tris-HCl (pH 8.0) , the N-lobe and C-lobe fractions were dialyzed against 50 mM Na acetate (pH 6.9), 1mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM $MnCl_2$ and applied to a ConA-Sepharose column (which binds glycosylated C-lobe but not N-lobe of bTf).Eluants from the column washed with the buffer just described were retained as the N-lobe containing fraction. The C-lobe containing fraction was eluted by using the same buffer containing 0.2 M methyl-a-D-mannopyranoside. Both C-lobe and N-lobe fractions were dialyzed against 50 mM Tris-HCl (pH 8.0), concentrated by ultrafiltration, and frozen as aliquots at 70°C.

Expression of recombinant receptor protein. *E. coli* strains carrying the appropriate recombinant plasmid (DH5αF/pCRIIPHtbpB for TbpB and DH5αF/pCRIIPHtbpA for TbpA) were used to

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inoculate 50 ml LB-broth starter cultures containing 0.2% maltose and 150 mg/ml ampicillin. After growth at 37°C for several hours the cultures were used to inoculate 1 liter of the same medium to a starting OD_{600} of 0.05. Once the OD_{600} reached 0.4, glucose was added to 4 mg/ml and grown until OD_{600} reached 0.7-0.8. At that time $MgSO_4$ to 10 mM and 100 ml of a 10^{10} pfu/ml suspension of CE6 l phage were added. The cell culture was incubated for an additional 2 hrs at 37°C and then harvested by centrifugation. The cell pellet was resuspended in 5 ml of ice cold 50 mM Tris-HCl pH 8.0, 1 M NaCl for affinity isolation, SDS-PAGE, and Western Immunoblot analysis.

Affinity isolation of transferrin binding proteins and analytical methods. Bovine transferrin was coupled to CNBr-activated Sepharose 4B (Pharmacia) according to the manufacturer's instructions using solutions containing 3.5 mg/ml of iron-saturated bTf. Activated groups were blocked by addition of ethanolamine. Non-coupled transferrin was removed by washing with 10 to 20 column volumes of a 50 mM TrisHCl, 1 M NaCl, pH 8.0 buffer containing 6.0 M guanidine hydrochloride. After further washing with 50 mM Tris-HCl (pH 8.0) the bound transferrin was reloaded with iron using a solution containing 5 mg/ml $FeCl_3$ in 0.1 M sodium citrate/0.1 M $NaHCO_3$ (pH 8.6). After washing again with 50 mM Tris-HCl (pH 8.0), the bTf-Sepharose resin was pre-equilibrated with 50 mM Tris-HCl (pH 8.0), 1 M NaCl before it was used in affinity experiments *E. coli* cells containing recombinant plasmids resuspended in 50 mMTris-HCl (pH 8.0)/1 M NaCl were solubilized in 20 mM EDTA, 2 % Sarkosyl and incubated for 2 h at room temperature. The mixture was centrifuged for 15 min at 8,000 rpm (4°C) and the supernatant containing the solubilized receptor was carefully decanted. The supernatant was diluted 4 times with 50 mM HCl (pH 8.0), 1 M NaCl buffer and pre-incubated for 30 min at room temperature with an excess (1 mg/ml) of each of the following transferrins diluted in the same buffer: iron-loaded bTf, goat or caprine transferrin (cTf), sheep or ovine transferrin (oTf), and human transferrin (hTf); apo-bTf; C-lobe bTf; and N-lobe bTf. Only the buffer was added to the positive control experiment. After pre-incubation the supernatants were applied to a bTf-Sepharose column previously equilibrated with 50 mM Tris-HCl (pH 8.0), 1 M NaCl and incubated for 15 min at room temperature. Each column was washed extensively with at least 12 column volumes of 50 mM Tris-HCl, (pH 8.0),1 M NaCl, 10 mM EDTA, 0.5% sarkosyl followed by 10 column volumes containing only 0.05% sarkosyl to remove non-specifically bound protein. Final wash was done with 50 mM Tris-HCl (pH 8.0), 0.5 M NaCl.

Elution of the recombinant TbpB was achieved by application of 1 bed volume of 2 x SDS-PAGE sample buffer under non-reducing condition and no boiling. Each eluant (supernatant) was collected after centrifuging the mixture containing the resin for 5 min at 13,000 x g in a microfuge. An aliquot of each supernatant (eluant) was further submitted to SDS-PAGE and electroblotted to Immobilon PVDF (Millipore) membrane (overnight at 15V in 20 mM Tris, pH 7.5, 150 mM glycine, 20% methanol and 0.1% SDS). The membrane was blocked with 0.5% skim

milk in 20 mM Tris (pH 7.5), 500 mM NaCl (TBS) for 30 minutes at room temperature. A 1/1,000 dilution of the anti-TbpB serum in the blocking solution was applied to the membrane for 1 hour at 37°C followed by two, 10-minute washes each with TBS. A 1/3000 dilution of secondary antibody (goat anti-rabbit IgG-horseradish peroxidase conjugate) was allowed to bind for 1 hour at 37°C. The conjugate was removed by three, 10-minute washes in TBS and developed using an HRP-substrate mixture (Chloro-Naphtol/H_2O_2).

N-terminal amino acid sequence analysis. Samples of affinity-purified and sequentially eluted TbpA and TbpB from H196 were subjected to SDS-PAGE, electroblotted onto PVDF (Immobilon-P, Millipore IPVH 00010) membrane, briefly stained with Coomassie Blue, and strips containing the individual protein bands were cut from the membrane for N-terminal amino acid sequence analysis.

Preparation of anti-TbpA and anti-TbpB monospecific rabbit sera. Approximately 500 mg of purified TbpA and TbpB from *P. haemolytica* strain H44 obtained from the appropriate fractions in the affinity procedure after dialysis and concentration was mixed with Freund's complete adjuvant and injected intramuscularly into two white female New Zealand rabbits, respectively. The rabbits were boosted twice at 3-week intervals with the same amount of antigens plus Freund's incomplete adjuvant. Two weeks after the final boost, blood was collected to determine the serum titre to the respective antigens using the dot assay in a dot-blot apparatus. The rabbits were either further boosted if titre was unsatisfactory or terminally bled, if the titre was satisfactory. The specificity of the sera against TbpA and TbpB from H44 was examined by SDS-PAGE and Western Immunoblot analysis using goat anti-rabbit IgG conjugated to HRP as secondary antibody. Expectedly, both TbpA and TbpB antisera crossreacted with TbpA and TbpB from strain H196, respectively.

Nucleotide sequence analysis. Two separate strategies for sequencing the tbp region were adopted. One approach primarily involved subcloning fragments from recombinant plasmids into the M13 vectors and then sequencing subsequently isolated single stranded DNA prepared by the dideoxy chain termination method using vector primers. In a limited number of cases, oligonucleotide primers were synthesized on the basis of the sequence results from the cloned inserts and used to complete the sequence of the cloned insert. In this analysis the nucleotide sequences were complied and analyzed by the Pustell programs (IBI).

An alternate approach primarily involved sequence determination of a succession of cloned inserts obtained by PCR amplification from chromosomal DNA. Oligonucleotide primers were synthesized on the basis of the preceeding sequence analysis. The PCR amplified products were cloned into the pCRII cloning vector (Invitrogen). Double stranded DNA sequencing was performed using purified recombinant plasmids by the oligonucleotide primer-directed procedure using synthetic oligonucleotides, fluorescent dye-labelled dideoxynucleotide triphosphate terminators, and cycle sequencing with Taq polymerase. Sequence reaction products were analysed on a Applied Biosystems(ABI) model 373A automated fluorescent sequencer. The

results from successive sequencing runs were compared and the composite sequence was determined by comparison of the chromatograms using the SeqEd program. This sequence was subsequently compared to the sequence obtained by single strand sequencing using the Mac-DNASIS program. In addition the sequence was analyzed by comparing the predicted protein sequence in all three reading frames with aligned sequences for Tbps from several different species. Any areas of uncertainty identified by this analysis were subjected to repeated runs of sequence analysis.

RESULTS

Cloning the transferrin receptor genes. Anti-receptor antisera and N-terminal amino acid sequences were obtained in order to facilitate cloning of the *P. haemolytica* transferrin receptor genes. Monospecific antisera was obtained by immunizing rabbits with the affinity purifed receptor proteins, TbpA and TbpB from a serotype A1 strain (H44) of *P. haemolytica*. Amino acid sequence analysis of an electroblotted preparation of purified native H196 TbpA yielded a readable sequence of 20 amino acids (top of Figure 20). A similar analysis with the purified TbpB failed to provide any sequence information suggesting the N-terminus of this protein may be blocked.

The sequence of the first eight amino acids of the purified TbpA was used to design an oligonucleotide primer based on a *P. haemolytica* preferred codon usage table (tbpA primer 023, Table 4). This primer was used in combination with either of two vector primers (RL2 and RL3, Table 4) for polymerase chain reaction (PCR) amplification of a portion of the tbpA gene from a *P. haemolytica* plasmid bank (Lo et al., 1985). An 800 bp PCR product was obtained with vector primers RL2 and 023 and its authenticity was verified by sequence analysis since the predicted amino acid sequence contained a sequence that was identical to the N-terminal amino acid sequence and exhibited homology with other TbpA proteins. The cloned PCR product was used as a hybridization probe for Southern analysis of restriction endonuclease-digested H196 *P. haemolytica* chromosomal DNA and for screening of the plasmid bank. The Southern analysis provided a restriction map of the chromosomal DNA in the tbp region for comparison to cloned inserts obtained from the plasmid bank.

(See also SEQ. ID. NOS. 25 to 37)

Table 4: Oligonucleotide primers.

Primer No.	Description (gene/region - location)	Direction*	Sequence
023	*tbpA* - 5'end, 1st 8 N-terminal aa's	5'-3'	GGAAGCTTACTGAAAATAAAAAAATC GAAGAA
088	*tbpA* - 5'end,	3'-5'*	CACTACTTTCCCCAAGCCAG
RL2	pBR322 - upstream of BamH1 site	3'-5'*	GGAATTCCCTCCTGTGGATC
198	*tbpA* - 3' end,	3'-5'*	GCIGCII(G/C)IGCICGIAA(T/C)T(T/A)(T/C)
190	*tbpB* - 5' end, leader peptide region	5'-3'	CAAAGCTTGCITG(TC)TCIGGIGG
352	upstream of *tbpB* - 5' end	5'-3'	AGATCTGGATTCTAAATCAGACCGCTTG TATTTTAG

192	*tbpB* - conserved aa sequence near 3' end	5'-3'	GTI(T/A)(A/G/C)IGGIGGITT(C/T)TA(T/C)GG
401	*tbpB* - 5' end	5'-3'	TAAATTAAAGGAGACATTATGTTTAAACT
350	*tbpB* - 3' end, flanking NcoI site	3'-5'*	CGACGCCCATGGTTATTTTTCTATTTGAC GTTTTCC
199	*tbpB* - 3' end, flanking HindIII site	3'-5'*	GCGCAAGCTTTTATTTTTCTATTTGACG
349	*tbpA* - 5' end, BamH1/BglII sites upstream of rbs	5'-3'	GGATTCAGATCTTAAAGGAGACCCTATC TAATGATAATG
255	*tbpA* - 5' end, NdeI site at start codon	5'-3'	CCCTATCATATGATAATGAAATATCATC
256	*tbpA* - 3' end, HindIII site after stop	3'-5'*	TAGCGCAAGCTTCTAAAACTTCATTTCAAAT

* Direction relative to orientation of coding strand for the relevant gene.

Initially, two strongly hybridizing colonies were identified from the *E. coli* clones. Plasmid p**(clone 9) contained a 9 kb insert which included most of the tbpA gene with adjacent downstream regions but was fused with DNA from another chromosomal locus (fis in Figure 20). The second plasmid, p**(clone 10), only contained a 1.2 kb insert that was primarily situated around the 5 end of the tbpA gene.

The artificial junction in plasmid pRYCL9 was reminiscent of similar artifacts observed while attempting to clone the meningococcal tbpB gene and the ensuing difficulties that were encountered (23) prompted consideration of alternative strategies for cloning the *P. haemolytica* tbpB region. One strategy was based on the observation that in other species the tbpB gene was located upstream of the tbpA gene (19, 20, 23) and that there were short stretches of amino acid identity in the predicted sequences of the respective TbpBs. A conserved amino acid sequence near the carboxy terminus of TbpBs was used to design a degenerate oligonucleotide primer (primer 192, Table 4) to obtain the remainder of the tbpA gene, the intergenic region and a portion of the 3' end of the tbpB gene. This primer was used in combination with a primer based on the sequence from the 5 end of the tbpA gene (primer 088, Table 4) to amplify a 700 bp fragment from H196 chromosomal DNA. The sequence from this insert enabled the design of an oligonucleotide primer based on the authentic sequence of the 3' end of the tbpB gene (primer 199, Table 4) which was used in combination with degenerate oligonucleotide based on a conserved amino acid sequence present in the leader peptide region of known TbpBs (oligo 190, Table 4) for PCR amplification. The resulting 2.4 kb PCR product obtained when H196 chromosomal DNA was used as a template contained the authentic 3' end of the tbpB gene. When this PCR fragment was cloned in the pCRII vector and used in expression experiments utilizing the T7 promoter, an intact recombinant TbpB was produced indicating that the ribosomal binding site and start of the tbpB gene was contained within the insert.

A second strategy utilized anchored PCR in which PstI-digested pBluescript plasmid was ligated to Pst I-digested H196 chromosomal DNA and used as template for a PCR reaction utilizing a primer from the 3' end of the tbpB gene (oligo 199, Table 4) and the M13 reverse primer from the vector. The resulting 3.5 kb product was subcloned into the PCRII vector,

producing a plasmid that contained the entire tbpB gene and a considerable amount of adjacent upstream regions (ORF and RNase T in Figure 20). and SEQ. ID. NO. 21

Further subcloning the tbp genes into expression vectors involved PCR amplification with oligonucleotide primers complementary to the 5 and 3 ends of the genes and inclusion of appropriate restriction sites. One set of primers for amplifying the tbpA gene (primer 349 and 256, Table 4) involved introduction of a BamHI and Bgl II site immediately upstream of the predicted ribosomal binding site (rbs) so that provision of an exogenous promoter should result in expression due to the presence of the native rbs. An alternate 5 primer (255, Table 4) involved the introduction of an Nde I site at the start codon so that cloning into the pT7-7 expression vector, which supplies a properly position ribosomal binding site, was possible. Since the expression experiments preceeded definitive identification of the start of the tbpB gene, the subcloned tbpB gene was obtained by PCR amplification with an upstream sequencing primer (primer 352, Table 4) and a primer containing the authentic 3 end flanked by an Nco I site (primer 350, Table 4).

Characterization of the transferrin receptor genes. As illustrated in Figure 20, the tbp genes appear to be in an operon arrangement, with the tbpB gene located upstream of the tbpA gene. Comparative sequence analysis revealed that the tbpB gene was preceded by an open reading frame (ORF) encoding a protein whose sequence was highly identical to RNaseT from *E. coli* and *H. influenzae*. This ORF was in turn preceeded by another ORF encoding a protein with considerable identity to hypothetical proteins identified in influenzae and *Vibrio parahaemolyticus*. Downstream of the tbpA gene is an ORF encoding a protein whose sequence was 70% identical to factor-for-inversion stimulation (FIS protein - recombinational enhancer) proteins from *H. influenzae* and *E. coli*. This effectively demarcates the boundaries of the transferrin receptor protein gene operon and indicates that there are no immediately adjacent genes related to this iron acquisition pathway.

There is a 420 bp region between the end of the ORF encoding the RNaseT homologue and the start of the tbpB gene with potential ribosomal binding sites, promoter sites and regulatory sites all present within the last 62 base pairs (Figure 20). The remaining 358 bp intervening region could presumably contain transcriptional termination signals for the RNaseT gene and sequences potentially involved in regulation of the tbp operon. The putative promoter region contains 5/6 and 6/6 of the consensus bases for the *E. coli* s70 -35 and -10 promoter regions, respectively.

Previous studies have demonstrated that the expression of the *P.haemolytica* transferrin receptor proteins is regulated by the level of available iron in the medium (26). The identification of a putative Fur box overlapping the -10 site of the tbpB promoter (Figure 20) suggests that regulation by iron may be at the transcriptional level via the action of a Fur homologue in *P. haemolytica*. The putative Fur box had 12/19 bases identical to the *E. coli* Fur binding site consensus sequence (Litwin and Calderwood, 1993).

Between the tbpB and tbpA genes there is a 96 bp intergenic region which contains a putative ribosomal binding site upstream of the tbpA gene but no evident promoter. In addition, there is no evident transcriptional terminators in this region. Downstream of the tbpA gene stop codon and the stop codon of the gene encoding the FIS homologue is a 98 bp region with no evident transcriptional terminators.

Sequence analysis of the tbpA gene region from serotype A1 *P. haemolytica* strain H196 revealed an ORF of 2,790 bp encoding a protein with a predicted molecular mass of 106,921 Da (Figure 22). The putative signal peptidase cleavage site at residue 28 was confirmed by comparison with the known N-terminal amino acid sequence of the mature protein (top of Figure 22). The predicted amino acid sequence of TbpA was compared to the sequences of TbpA from *N. meningitidis* (23), *N. gonorrhoeae* (8), *H. influenzae* (20) and *Actinobacillus pleuropneumoniae* (19). The localization of identical amino acids between these proteins (bold-underlined amino acids, Figure 22) was compared to the proposed topology of these amino acid segments based on the model predicted by Tommassen (28). It is evident that most of the identical amino acids are clustered in regions corresponding to the short transmembrane β-sheets or in the segments of the internal and external loops that are immediately adjacent to the transmembrane sections. It is interesting to note that there are conserved pairs of cysteines in proposed external loops 4, 6 and 7 and a unique cysteine pair in loop 10 of the *P. haemolytica* TbpA. These likely represent disulfide bridges that would provide structural stability to the external loops.

Analysis of the sequence in the tbpB gene from serotype A1 *P. haemolytica* strain H196 revealed an ORF of 1,752 bp encoding a protein with a predicted molecular mass of 63,419 Da (Figure 23). This predicted protein sequence of TbpB was compared to the published sequences of TbpBs from *N. meningitidis* (23), *N. gonorrhoeae* (1), *H. influenzae* (20) and *Actinobacillus pleuropneumoniae* (14). This predicted amino acid sequence included a 18 amino acid leader peptide, a signal peptidase II recognition sequence with a cysteine as the predicted N-terminal amino acid of the mature protein. The presence of an N-terminal cysteine, which has been shown to be lipidated in other species (14, 24), may explain the inability to obtain an N-terminal amino acid sequence for this protein and may serve as the primary means of anchoring the protein to the outer membrane. Regions that aligned with the putative binding regions of the *A.pleuropneumoniae* TbpB (TfbA) recently identified by Gerlach et. al. (36) are indicated by a double underline.

It is apparent that there are several regions of homology found throughout the length of the amino acid sequence which include several short stretches of identical amino acids (Figure 24). Upon closer inspection it is apparent that there is some homology between regions in the N-terminal and C-terminal portions of the protein suggesting there may be an underlying bilobed structure to the protein, analogous to what is observed for transferrins. Thus the sequences YKGYW (aa 185-189) (SEQ. ID. NO. 38) and YRGTW (aa 449-453) (SEQ. ID. NO. 39), FTADFANK (aa 237-244) (SEQ. ID. NO. 40) and

FDVDFVNK (aa 480-487) (SEQ ID NO:41), GNRFSG (aa 276-281) (SEQ. ID. NO:42) and GNGFGG (aa 513-518) (SEQ. ID. NO:43), and LEGGFFG (aa 300-306) (SEQ. ID. NO:44) and FEGGFYG (aa 546-552) (SEQ ID NO. 45) represent consecutive stretches of homologous amino acids in equivalent positions of the N-terminal and C-terminal portions of the protein.

Expression and analysis of the recombinant receptor proteins. The intact tbpB and tbpA genes were PCR amplified from H196 chromosomal DNA and subcloned into expression vectors for production of recombinant proteins. For initial attempts at expression of the tbpB gene, the subcloned tbpB gene was obtained by PCR amplification with an upstream primer (352, Table 4) and a primer containing the authentic 3 end flanked by an NcoI site (350, Table 4). When the PCR amplified fragment was subcloned into the pCRII vector, all five of the resulting clones were in the same orientation; downstream of the T7 promoter and in the opposite direction of the lac promoter. Since the lac promoter would not be tightly regulated in a high copy number vector, this result suggests that expression of the insert in *E. coli* may be selected against. Once the sequence of this region was available it became apparent that primer 352 was immediately upstream of the RNaseT gene and thus expression of either this gene or the tbpB gene could have been responsible for the selective pressure. Expression of TbpB from the T7 promoter was accomplished by infection with CE6 λ phage, which encodes the T7 RNA polymerase. Two hours after infection a protein of the anticipated molecular weight for TbpB was evident and this protein reacted with anti-TbpB antiserum after electroblotting.

There was detectable binding of labelled bovine transferrin (bTf) by immobilized intact cells expressing the TbpB protein and this level of binding did not increase significantly with prior sonication of the cells (data not shown). Although this could be interpreted as proper processing and export of the TbpB to the cell surface in the heterologous *E. coli* system, disturbance of the outer membrane integrity by overexpression of a foreign protein antigen is an equally plausible explanation. The preliminary binding studies suggested that a functional TbpB protein was being produced, indicating that further analysis might enable evaluation of the functional properties of this protein and ascertain its contribution to the previously characterized properties of the native receptor complex. Thus, crude membranes were prepared from cells expressing TbpB and used in affinity isolation experiments designed to evaluate its binding characteristics. These experiments indicated that the recombinant TbpB is capable of being affinity isolated by immobilized bTf and this isolation could be inhibited by an excess of bovine, ovine or caprine Tf, demonstrating its ability to effectively bind to all three of these ruminant Tfs. Human transferrin as well as apo-bTf did not inhibit the affinity isolation of recombinant TbpB by immobilized iron-loaded bTf. This assay also revealed that both N-lobe and C-lobe of bTf effectively blocked binding of recombinant TbpB to the immobilized bTf.

For expressing the tbpA gene, PCR amplification was performed with a set of primers (oligos 349 and 256, Table 4) that maintained the predicted ribosomal binding site (Figure 21). After subcloning the PCR product into the pCRII vector, no recombinant TbpA was expressed after infection with CE6 phage (encoding T7 RNA polymerase). Sequence analysis of

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one of the clones revealed excision of several base pairs that eliminated ribosomal binding site. Other repeated attempts at subcloning the tbpA gene from PCR using 255 and 256 primers (see Table 4) into the NdeI site of the pT7-7 vector (which provides an optimally positioned ribosomal binding site) were unsuccessful.

Discussion

In the initial studies demonstrating the presence of a transferrin receptor in *Pasteurella haemolytica* (26) only a single receptor protein (TbpA) was isolated by an affinity method that yielded two receptor proteins (TbpA and TbpB) in other species (32). Thus the ability to use iron from bovine transferrin for growth (26) and the specific binding of ruminant transferrins (39) was initially presumed to be largely mediated by this receptor protein. In subsequent studies that demonstrated that the binding region of bovine transferrin was localized to the C-lobe (41), two receptor proteins (TbpA and TbpB) were isolated by a modified affinity method, although the yield of TbpA greatly exceeded that of TbpB. Thus it was not possible to conclusively attribute the observed binding characteristics of the receptor to either receptor protein, and particularly not to TbpB.

The cloning of the tbp genes and expression of recombinant TbpB has enabled specific evaluation of its binding characteristics. These studies have demonstrated that TbpB has a similar host specificity as the native receptor complex (TbpA and TbpB) as it specifically binds Tfs from several ruminant species. In contrast, unlike the native receptor complex, the recombinant TbpB was able to recognize binding determinants on the N-lobe as well as the C-lobe of bTf, suggesting that in the previous study (Yu and Schryvers, 1994) an interaction between TbpA and TbpB might have interfered in the ability of TbpB to also bind to the N-lobe of bTf.

In competitive binding assays with immobilized membranes (TbpA and TbpB) from *P. haemolytica* there was no evident preference for the iron-loaded or apo form of bTf (30) which is similar to what has been observed in most other bacterial species (Blanton et al., 1990; 32; Tsai et al., 1988; 32, 37), except *Moraxella catarrhalis* (Yu and Schryvers, 1993). In the present study recombinant TbpB clearly showed a strong preference for the iron-loaded form of bTf. This preference of TbpB may have functional relevance in increasing the efficiency of iron acquisition *in vivo*.

Example 4

Vaccine Potential of Recombinant Tbp2 and Authentic Tbp1

The transferrin-binding proteins Tbp1 and Tbp2 are attractive targets for a number of reasons:

a) Since acquisition of iron from transferrin is likely essential for bacterial survival, an antibody response against these antigens should be protective.

b) The genes coding for Tbp1 and Tbp2 appear to be conserved within various isolates of *P. haemolytica* A1.

This study deals with testing the vaccine potential of recombinant Tbp2 and authentic Tbp1, alone and in combination, in an experimental *P. haemolytica* challenge model.

Methods

The Tbp1 and Tbp2 proteins were affinity purified from *P. haemolytica* and recombinant *E. coli* outer membranes, respectively, by affinity chromatography using standard techniques as described herein. Vaccines were formulated using a proprietary mineral oil-based adjuvant (VSA3) such that the volume of each dose was 2 cc containing the following amount of each antigen: Tbp2 - 45 mg; Tbp1 - 85 mg when used by itself or 100 mg when combined with Tbp2. In addition, a placebo vaccine was prepared containing sterile diluent in place of antigen. Five groups were included in the trial, including one which received a single immunization of Tbp2 ten days before challenge, and groups which received two immunizations with Tbp2, Tbp1 + Tbp2, placebo, or Tbp1. The interval between primary and secondary immunization was three weeks and all vaccinations were carried out at a farm in Southern Saskatchewan. Vaccines were delivered via the subcutaneous route. Approximately ten days prior to challenge, animals were transported to Saskatoon and housed at the VIDO research station. All groups contained ten animals with the exception of the group receiving Tbp1 in which there were six. This group was not in the original proposal and was added in order to determine the protective capacity of Tbp1 by itself. In addition, one calf which received one immunization with the Tbp2 formulation developed clinical signs of disease unrelated to vaccination and was therefore excluded from the trial. The composition of the vaccine groups is summarized in Table 5.

Table 5. Composition of vaccine groups.

Vaccine Group	Antigens	Immunizations	Animals/Group
1	Tbp2	One	9
2	Tbp2	Two	10
3	Tbp1 & Tbp2	Two	10
4	Placebo	Two	10
5	Tbp1	Two	6

Calves were challenged via the aerosol route by first exposing them to a suspension of bovine herpesvirus-1 strain 108 containing approximately 2.5×10^6 PFU/ml followed four days later by an aerosol of *P. haemolytica* containing approximately 5×10^8 CFU/ml. Animals were examined daily by a veterinarian and animal health technician and the following data was recorded: weight, temperature, nasal scores, depression, strength, respiratory distress and sickness. Each of these criteria with the exception of weight and temperature were scored on a scale of 0-4.

The serological response to vaccination was measured using an enzyme-linked immunosorbent assay (ELISA). Serum samples were collected at the time of the first and second immunizations plus on the day of challenge with BHV-1. The titers are presented as the reciprocal of the serum dilution which resulted in an optical density equivalent to the background plus two standard deviations. Responses against Tbp1, Tbp2 and the *P. haemolytica* leukotoxin were measured. The latter was included as a diagnostic test to determine if animals had been naturally exposed to the organism.

Results

a) Response to Vaccination: None of the animals showed any adverse reaction to vaccination with any of the formulations used. The serological response to vaccination was determined using an ELISA procedure which measured the serum antibody levels to Tbp1, Tbp2 and the *P. haemolytica* leukotoxin. The latter antigen was included in order to ensure that none of the animals had increased titers due to the natural exposure to the bacteria. The titers against each antigen are shown in Table 6 and it can be seen that the titers against leukotoxin were comparable at the time of initial vaccination (Bleed 1), the second vaccination (Bleed 2), and at challenge (Bleed 3). Animals which have a titer below 3,000 were considered to be clean. Interestingly, none of the animals seroconverted to a significant degree to the Tbp1 antigen. Based upon the inventors experience with Tbp1 from other organisms, the expected titers should be low but it was unexpected that no significant increase in antibody levels would be detectable. All groups which received Tbp2 responded well to vaccination and although the group which received Tbp1 + 2 had titers approximately 1/2 of that in the Tbp2 group, this difference is not significant.

Table 6. Serological response to vaccination.

ELISA Antigen	Vaccine Group	Titer - Bleed 1	Titer - Bleed 2	Titer - Bleed 3
Leukotoxin	Tbp2 (1 dose)	1010	1350	2156
	Tbp2	1136	1547	2656
	Tbp1 & 2	1159	1239	1137
	Placebo	1688	1834	2782
	Tbp1	980	1282	1339
Tbp1	Tbp2 (1 dose)	113	364	229
	Tbp2	203	206	296
	Tbp1 & 2	150	388	367
	Placebo	140	130	226
	Tbp1	57	55	139
Tbp2	Tbp2 (1 dose)	408	639	9871
	Tbp2	397	12154	66697
	Tbp1 & 2	119	7838	27515
	Placebo	360	269	378
	Tbp1	359	433433	478

ELISA titers against leukotoxin, Tbp1 and Tbp2 were determined using serum samples taken at the time of the first immunization (Bleed 1), the second immunization (Bleed 2), and the day of challenge with bovine herpesvirus-1 (Bleed 3). The numbers are expressed as the reciprocal of the dilution equaling a negative control plus two standard deviations.

b) Mortality: The experimental disease model has been calibrated to obtain 60-70% mortality under normal conditions. However, mortality was higher than usual in this trial, probably because of the extremely cold temperatures to which the animals were exposed throughout the time period after challenge. Daily low temperatures were in the -40°C range and all animals were housed outdoors during the trial. The mortality by group is shown in Table 7 and the only group which showed significant protection was that which received both Tbp1 and Tbp2. This is compared to 50% mortality for Tbp2 by itself and 100% for Tbp1. A single immunization with Tbp1 was of no benefit.

Table 7. Group mortality observed during the trial.

Vaccine Group	Mortality (%)
Tbp2 (1 dose)	78
Tbp2	50
Tbp1 & 2	10
Placebo	90
Tbp1	100

c) Clinical Signs of Disease: The clinical results are summarized by group in Table 8. It should be noted that Table 8 contains the clinical results for all days of the trial, including those prior to *P. haemolytica* infection on day four. Therefore, only the results from days 4 through ten have been used to determine the protective capacity of the vaccine formulations. The high rate of mortality observed during this trial had the effect of reducing the size of each group to the point where differences observed in all of the clinical parameters measured are not statistically significant. However, it is clear that the group which received both Tbp1 and Tbp2 showed lower scores in most categories between days 5 and 7. The groups which received two immunizations with Tbp2 alone also showed reduced clinical signs of disease in survivors although they did not do as well as the combination group. The contribution of Tbp1 to protection is unclear at present since there did not appear to be any antibody response to this antigen.

d) Postmortem Results: Necropsies were performed on all animals which died during the trial. In all cases, *P. haemolytica* was cultured from the lungs and the pathology observed was consistent with fibrinous pneumonia caused by *P. haemolytica.*

Conclusions

Two vaccinations with a formulation containing *P. haemolytica* Tbp1 and Tbp2 provided significant protection against experimental bovine pneumonic pasteurellosis. The exact contribution which Tbp1 provides to this protection is unclear since there did not appear to be any serological response to the protein. The beneficial effect may have been due to a cell-mediated immune response.

Immunization with two doses of Tbp2 provided some degree of protection and it may be possible to increase this by testing vaccine formulations containing greater quantities of antigen or a different adjuvant. It is likely that the immunological response to Tbp2 provided the bulk of the protection seen with the combination vaccine.

Vaccination with one dose of Tbp2 or two doses of Tbp1 had no beneficial effect after experimental challenge.

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Table 8

Mean Clinical Scores by Group. Animals were challenged on day 0 with BHV-1 and day 4 with *P. haemolytica*.

Mean Weight (kg)

Group	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10
Tbp2 (1 dose)	232.4	231.6	228.1	225.9	222.9	223.1	223.6	225.7	256.0	256.0	256.0
Tbp2	211.4	209.3	205.3	202.4	201.2	191.5	197.3	195.5	198.8	197.7	197.2
Tbp1 & 2	217.8	218.1	216.0	212.9	211.2	208.8	207.5	208.3	207.7	207.8	208.3
Placebo	223.1	221.2	215.7	213.6	209.5	206.2	194.4	177.0	158.0	161.0	158.0
Tbp1	225.3	225.8	219.8	216.7	212.7	208.2	200.0	193.0	191.0	190.0	N/A

Mean Temperature

Group	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10
Tbp2 (1 dose)	39.06	38.96	39.92	40.21	40.53	40.00	39.40	39.50	39.25	38.85	39.30
Tbp2	39.05	39.26	40.29	40.50	40.44	40.51	40.19	39.67	39.88	39.33	39.10
Tbp1 & 2	39.19	39.36	40.10	40.00	40.21	40.20	39.48	39.49	39.14	39.26	39.48
Placebo	39.03	39.10	40.30	40.41	40.83	40.24	39.96	40.35	39.50	39.10	40.70
Tbp1	39.15	38.78	40.52	40.12	40.47	40.85	40.95	41.60	40.90	41.00	N/A

Mean Nasal Score

Group	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10
Tbp2 (1 dose)	0.00	0.11	0.33	0.61	1.17	1.33	1.40	1.25	0.00	0.00	0.00
Tbp2	0.00	0.05	0.30	0.85	1.20	1.45	1.56	1.44	0.83	0.33	0.20
Tbp1 & 2	0.00	0.10	0.45	0.60	1.05	1.70	0.90	1.00	0.70	0.33	0.44
Placebo	0.00	0.15	0.35	0.95	1.10	1.78	1.40	1.50	1.00	0.00	0.00
Tbp1	0.00	0.08	0.33	0.67	0.83	1.08	1.00	0.00	1.00	2.00	N/A

Mean Depression Score

Group	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10
Tbp2 (1 dose)	0.00	0.00	0.00	0.11	0.22	0.67	1.60	1.75	0.00	0.00	0.00
Tbp2	0.00	0.00	0.00	0.15	0.00	0.50	0.89	1.67	0.67	1.33	0.40
Tbp1 & 2	0.00	0.00	0.00	0.00	0.00	0.60	1.20	1.10	0.60	0.33	0.56
Placebo	0.00	0.00	0.05	0.15	0.60	1.33	2.20	2.00	1.00	0.00	0.00
Tbp1	0.00	0.00	0.00	0.17	0.50	1.83	1.50	2.00	2.00	3.00	N/A

Mean Strength Score

Group	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10
Tbp2 (1 dose)	0.00	0.00	0.00	0.00	0.00	0.67	1.60	1.75	0.00	0.00	0.00
Tbp2	0.00	0.00	0.00	0.00	0.00	0.40	0.78	1.67	0.50	1.00	0.40
Tbp1 & 2	0.00	0.00	0.00	0.00	0.00	0.30	0.50	1.10	0.60	0.22	0.11
Placebo	0.00	0.00	0.00	0.00	0.00	0.67	1.60	1.50	1.00	0.00	0.00
Tbp1	0.00	0.00	0.00	0.00	0.00	1.17	0.50	1.00	2.00	3.00	N/A

Mean Respiratory Distress Score

Group	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10
Tbp2 (1 dose)	0.00	0.00	0.00	0.00	0.00	0.11	1.40	2.00	0.00	0.00	0.00
Tbp2	0.00	0.00	0.00	0.00	0.00	0.10	0.44	1.22	0.33	0.67	0.00
Tbp1 & 2	0.00	0.00	0.00	0.00	0.00	0.00	0.30	0.40	0.50	0.11	0.11
Placebo	0.00	0.00	0.00	0.00	0.00	0.67	1.00	1.00	0.00	0.00	0.00
Tbp1	0.00	0.00	0.00	0.00	0.00	1.17	0.50	1.00	1.00	3.00	N/A

Mean Sick Score

Group	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10
Tbp2 (1 dose)	0.00	0.00	0.56	0.78	0.89	0.89	2.00	2.25	0.00	0.00	0.00
Tbp2	0.00	0.00	0.90	0.90	1.00	1.00	1.00	1.89	0.83	1.17	0.40
Tbp1 & 2	0.00	0.00	0.60	0.50	0.90	1.10	0.90	1.20	0.70	0.33	0.44
Placebo	0.00	0.00	0.70	1.00	1.20	1.80	2.71	2.67	1.00	0.00	1.00
Tbp1	0.00	0.00	0.83	0.50	1.00	2.83	2.75	3.00	2.00	4.00	N/A

Cumulative Mortality

Group	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10
Tbp2 (1 dose)	0.00	0.00	0.00	0.00	0.00	0.00	0.44	0.78	0.78	0.78	0.78
Tbp2	0.00	0.00	0.00	0.00	0.00	0.00	0.10	0.30	0.40	0.40	0.50
Tbp1 & 2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.10	0.10
Placebo	0.00	0.00	0.00	0.00	0.00	0.40	0.50	0.80	0.80	0.90	0.90
Tbp1	0.00	0.00	0.00	0.00	0.00	0.33	0.67	0.67	0.83	0.83	1.00

EXAMPLE 5

Comparison of the transferrin receptor from various ruminant serotypes

A collection of *Pasteurella haemolytica* and *P. trehalosi* strains of various serotypes isolated from cattle,sheep and goats were analyzed for binding of ruminant transferrins and utilization of transferrin iron for growth. Some of the goals of the study were to determine the prevalence of transferrin receptors from different host species, to evaluate their specificities for different ruminant transferrins and to determine if there is antigenic relatedness amongst the surface receptors from the different strains causing shipping fever in cattle, pneumonia in sheep and goats and septiceamia in lambs.

Materials

Bacterial strains. The bacterial strains used in this study are listed in Table 9. Clinical type A1 isolates of *P.haemolytica* (h93-h97)(9) and representative ATCC strains (h98-h107) from bovine pneumonia were provided by Dr. Andrew Potter, VIDO, Saskatoon. *P. trehalosi* strain h174, field isolate from goat with pneumonic pasteurellosis was provided by Dr. Frank Milward, Rhone Merieux, Lyon, France. *P. haemolytica* strain h44 , a bovine clinical type A1 isolate from bovine pneumonia, has been described previously (26). Strain h196 was obtained from Dr. Lo,University of Guelph , Ontario, Canada.

Table 9. Bacterial strains, serotypes and sources.

Species	Strain	Serotype	Source
P. haemolytica	h44	A1	cattle
P. haemolytica	h93	A1	cattle
P. haemolytica	h94	A1	cattle
P. haemolytica	h95	A1	cattle
P. haemolytica	h96	A1	cattle
P. haemolytica	h97	A1	cattle
P. haemolytica	h196	A1	cattle
P. haemolytica	h98 (ATCC33366)	A2	sheep
P. trehalosi	h99 (ATCC33367)	T3	sheep
P. trehalosi	h100 (ATCC33368)	T4	sheep
P. haemolytica	h103 (ATCC33372)	A8	sheep
P. haemolytica	h104 (ATCC33373)	A9	sheep
P. haemolytica	h105 (ATCC33369)	A5	sheep
P. trehalosi	h106 (ATCC33374)	T10	sheep
P. haemolytica	h107 (ATCC33375)	A11	goat
P. trehalosi	h174 (90020-16266)	T3	goat

Growth conditions. All bacterial strains were stored frozen at -70C in 30% glycerol. Isolates from the frozen stocks were streaked onto chocolate agar plates and incubated at 37C in a 5% CO_2 incubator. Iron-restricted growth was achieved by growing the bacteria in Brain Heart Infusion broth (BH1, Difco Laboratories) or O'Reilly-Niven broth (25) supplemented with 3.0 g/ml nicotinamide adenine dinucleotide (NAD) and containing the iron chelator ethylenediaminedihydroxyphenylacetic acid (EDDA, Sigma) at a final concentration of 100 M. Growth experiments for use of different transferrins as an iron source was performed as previously described (26).

Preparation of transferrins and derivatives. Bovine transferrin was obtained from Sigma. The preparation of ovine (sheep) and caprine (goat) transferrins (2), the iron loading of transferrins to 30% or 100% saturation (Herrington et al.1985) and conjugation of horse-radish peroxidase (HRP) to transferrin (37) was essentially as described previously. In the preparation of conjugates of bovine, ovine and caprine transferrins (HRP-bTf, HRP-oTf and HRP-gTf), the mixture of HRP and transferrin were subjected to gel filtration after chemical conjugation. The fractions demonstrating maximal activity were pooled, dialyzed and aliquots frozen and stored at -70^{0}C.

Transferrin binding assay. The solid phase binding assay for transferrin was essentially as described previously (32). After the membrane or concentrated eluates were spotted unto HA paper (Millipore Corporation, Bedford, MA) and blocked in 0.5% skim milk, the paper was exposed to blocking solution containing 450 ng/ml of the HRP-conjugated transferrin. The incubation, washing and development with HRP substrate mixture were performed essentially as previously described (32).

Affinity isolation of transferrin binding proteins . Bovine, ovine and caprine transferrins were individually coupled to CNBr-activated Sepharose 4B according to the manufacturers instructions using solutions containing 3.5 mg/ml of iron-saturated transferrin. Activated groups were blocked by addition of ethanolamine. Non coupled transferrin was removed by washing with 10 to 20 column volumes of a 50 mM TrisHCl, 1 M NaCl, pH 8.0 buffer containing 6.0 M guanidine hydrochloride and after further washing the bound transferrin was reloaded with iron using a solution containing 5 mg/ml $FeCl_3$ in 0.1 M sodium citrate/0.1 M $NaHCO_3$ pH 8.6 buffer.

Iron-deficient total membrane (200 mg protein) from *P. haemolytica* or *P.trehalosi* prepared as previously described (32) was diluted to 2 mg/ml in 50 mM Tris pH 8.0 containing 1.0 M NaCl. The diluted membrane was solubilized by addition of EDTA and sarkosyl to a final concentration of 10 mM and 0.75%, respectively followed by incubation of the mixture at room temperature for 15-30 min with gentle rocking. The solution was centrifuged at 10,000 rpm for 10 min to remove insoluble debris. The supernatant containing the solubilized membrane was applied to a 1.5 x 10 cm transferrin-affinity column and then washed extensively (at least 10 bed volumes) with 50 mM Tris pH 8.0 containing 1.0 M NaCl, 10 mM EDTA, 0.75% Sarksosyl to

remove non-specifically bound protein. In experiments using low salt washing conditions the washing buffer contained 100 mM NaCl in lieu of 1M NaCl. In some instances, additional washing with 2-3 bed volumes of washing buffer containing 0.2 M guanidine hydrochloride was necessary to remove contaminating proteins.

Coelution of both transferrin binding proteins (TbpA and TbpB) was achieved by application of 2-3 bed volumes of 2.0 M guanidine hydrochloride in 50 mM Tris pH 8.0, containing 1.0 M NaCl, 1 mM EDTA, 0.01% sarkosyl. The eluant was collected for immediate dialysis against 50 mM Tris pH 8.0. Further treatment with higher concentrations of guanidine hydrochloride usually did not result in any further yield of receptor protein. Individual isolation of TbpA and TbpB was attained by sequential elution with 2 bed-volumes of each buffer containing 0.2, 0.5, 0.75, 1.0, 1.5, 2.0 and 3.0 guanidine hydrochloride, respectively. The eluates were dialyzed against 3 changes of 3 litres 50 mM Tris pH 8.0 over an 18-hour period and concentrated by ultrafiltration. After SDS-PAGE analysis the fractions from the 0.5 and 0.75 M guanidine HCl elution buffers were found to contain TbpB only and were thus pooled for a preparation of TbpB and fractions from the 1.5 and 2 M guanidine HCl elution buffers were pooled for a preparation of TbpA.

Preparation of anti-TbpA and anti-TbpB monospecific rabbit sera. Approximately 500 μg of purified TbpA or TbpB from *P. haemolytica* strain h44 prepared as described above, was mixed with Freund's complete adjuvant and injected intramuscularly into two white female New Zealand rabbits. The rabbits were boosted twice at 3-week intervals with the same amount of antigens in Freund's incomplete adjuvant and the immune sera collected 2weeks after the final boost. The specificity of the sera against TbpA and TbpB was tested after SDS-PAGE and immunoblotting of the receptor proteins and using goat anti-rabbit IgG conjugated to HRP as secondary antibody.

Analytical methods. Protein samples were analyzed by SDS-PAGE followed by silver staining as previously described (32). For Western blot analysis, about 1-2 μg of purified receptor proteins or 40 μg of outer membrane protein from iron-limited cells were separated on 10% polyacrylamide gels. Proteins were electrophoretically transferred to nitrocellulose (Millipore, Bedford, MA) overnight at 15V in 20 mM Tris, pH 7.5, 150 mM glycine, 20% methanol and 0.1% SDS. The filters were blocked with 0.5% skim milk in 20 mM Tris pH 7.5, 500 mM NaCl (TBS) for 30 minutes at room temperature. The membrane was exposed to 1/1000 dilution of the appropriate antibody in blocking solution for 1 hour at room temperature,washed twice with TBS, and then exposed to a 1/3000 dilution of secondary antibody (goat anti-rabbit IgG-horse-radish peroxidase conjugate from BioRad). The conjugate was removed, washed three times with TBS and then developed using the HRP-substrate mixture. For the whole cell assay, iron-deficient or iron-sufficient (control) cells were directly spotted onto HA paper. After drying, the HA paper was treated with blocking solution and washed with TBS and then tested for reactivity with anti TbpA or anti TbpB antisera as described above. A control set of spotted

cells was treated with HRP-bovine transferrin for 1 hour followed by development with HRP-substrate after washing in TBS.

PCR amplification of tbp genes and restriction endonuclease digest analysis. Amplification of *tbpA* and *tbpB* from *P. haemolytica* and P. *trehalosi* strains was performed on intact cells by the method of Saris et al.(1990). Amplification of tbpA was carried out with oligonucleotides *tbpA* 5' and *tbpA* 3' (#255 and #256, table2). Oligonucleotides #401 and # 199, (Table 10) were used to amplify *tbpB*. Reaction conditions consisted of 30 cycles of 94°C for 1min, 45°C for 1min, and 74°C for 2min. The PCR product was separated by 1% agarose gel electrophorsis in 0.5X TBE buffer (45mM Tris-borate, 1mM EDTA, pH8.3) and stained with 0.5mg of ethidium bromide per ml in the same buffer. For Sau3A restriction endonuclease (Gibco BRL) digestion, PCR products were subjected to phenol chloroform and ethanol precipitation and thereafter were digested with Sau3A. The digests were analysed with a 7.5% acrylamide gel run in 0.5X TBE buffer and visualised in the same way as the agarose gels described above.

Results

Specificity of receptor binding. In a prior study it was demonstrated that transferrin receptors in representative isolates from pathogenic bacterial species varied in their interaction with goat, sheep and cattle transferrin (Yu and Schryvers 1996). Thus a set of representative isolates of *P. haemolytica* and *P. trehalosi* from cattle, sheep and goats (Table 9) were evaluated for their interactions with the different ruminant transferrins. All of these strains were capable of utilizing bovine, caprine or ovine transferrin as a source of iron for growth (data not shown). Immobilized, iron-deficient cells were positive for binding all three of the transferrins in a solid-phase binding assay (Figure 25) and the three transferrins were equally effective at blocking binding to the cells in reciprocal competition binding assays (not shown). In addition, both TbpAand TbpB, molecular weights 100Kda and 60Kda respectively were effectively isolated by affinity resins containing immobilized bovine (Figure 26, Panel A), caprine or ovine transferrin (not shown). These results indicate that the specificity of transferrin-binding within this group of related strains is indistinguishable.

Immunological analysis of transferrin receptor proteins. The observation that bovine, caprine and ovine transferrins compete for the same receptors suggested that there is conservation at least in the binding domain of the receptors. However, the extent of similarity between the individual receptor proteins from different serotypes was not known. To address this question, antibodies were raised against TbpA and TbpB individually and as a complex in rabbits using purified receptor proteins (TbpA and TbpB from a bovine strain h44).These antisera were then tested against receptor proteins from representative strains of different serotypes including isolates obtained from cattle, sheep and goats.

The results in Figure 26, Panel B demonstrate that the antisera raised against purified TbpA and TbpB receptors from *P. haemolytica* serotype A1, strain h44, reacted strongly with similar purified receptors from all of the representative strains. Since there was variation in

the yield of receptor proteins obtained from the various strains (Figure 26, Panel A), this likely accounts for the slight differences observed in the reactivity of the antisera for a few of the strains (Figures 26, Panel B). Thus, these results suggest that both receptor proteins are conserved amongst the different serotypes of *P. haemolytica* causing pasteurellosis in cattle, sheep and goats.

Although this analysis indicates that there are crossreactive epitopes on the transferrin receptor proteins it does not provide any information as to whether there are crossreactive epitopes at the cell surface where they would be accessible to host immune effector mechanisms. As a first step in attempting to address this question the reactivity of intact iron-deficient cells from the different species to monospecific anti-receptor antisera was tested (Figure 27). These experiments demonstrated that the monospecific antisera prepared against TbpA and TbpB from a type A1 strain of *P. haemolytica* reacted with type A1 strains (h44 and h196, Figure 26, Panel B), several other A serotypes (h98, h103 , h105, and h107), and reacted moderately with several *P. trehalosi* strains (h99, h100, h106). The monospecific antisera prepared against TbpA and TbpB from strain h44 reacted strongly with iron-limited whole cells from an extended collection of type A1 strains (data not shown), two of which (h44, h196) are illustrated in Figure 27. There were varying degrees of reactivity with the other serotypes within *P. haemolytica* (h98, h103, h105 and h107) and *P. trehalosi* (h99, h100 and h106). The correlation between the signal obtained with the labelled bTf (Figure 27) and with the anti-TbpA and anti-TbpB antisera (Figure 27) in the different strains suggests that the observed reactivity in intact cells is primarily due to the receptor proteins. The absence of reactivity observed when control antiserum is utilized (data not shown) and the reduced reactivity in iron deficient cells (Figure 26, Panel C) also supports this conclusion.

Genetic analysis of transferrin receptor protein genes. As a complement to the immunological studies the variability of the *tbp* genes from the various strains of *P. haemolytica* and *P. trehalosi* was evaluated. Using the sequence information obtained for the *tbpA* gene from a serotype A1 strain (28), specific primers were prepared for the 5 and 3 ends of the gene (primer #255 and #256, Table 10). These primers were capable of amplifying the intact *tbpA* genes from all the tested strains although only small yields were consistently obtained for strain h100. The intact genes (except from h100) were then subjected to digestion by the Sau3A restriction endonuclease and the resulting fragments were analyzed by electrophoresis on polyacrylamide gels. A specific pattern was observed in type A1 strains (h44 and h196, Figure 28, Panel A) and was identical in all the seven type A1 strains that were tested (data not shown). This pattern was also present in most of the other serotypes within *P. haemolytica* (h105, h104, and h98, Figure 28). Subtle changes in the pattern, involving one or more fragments, that is likely attributed to alteration of a single site, was observed for other type A strains (h103 and h107) and within the T strains (h106 and h99).

The restriction digestion analysis was unable to identify any differences between the *tbpA* genes in the A1 isolates. Thus, an alternative approach was adopted for rapidly and simply examining variation amongst the *tbpA* genes. This approach is based on the observation that there are segments of the TbpA (and LbpA) protein, proposed to be surface loops, which show the greatest variation in amino acid sequence among proteins from different species (21 and Legrain et al.1996). In particular, there is one large predicted loop, demonstrated to be at the surface by reactivity with a monoclonal antibody (21), in which the greatest variation in amino acid sequence was observed when aligning TbpAs from two meningococcal strains , one gonococcal strain and four *H.influenzae* strains (1; Loosemore et al.1996;Schryvers and Gonzalez 1996). An oligonucleotide primer was prepared (#450,Table 10) based on the known amino acid sequence (VEDTCPTLD) in this region for *P. haemolytica* typeA1 and used it in colony PCR amplification reactions in combination with a 5 specific primer (#255 , Table 10) with the various strains. As illustrated in Figure 29, Panel A, this oligonucleotide pair readily amplified the anticipated 800 bp PCR product under high stringency conditions from all of the strains except strain h100 where a comigrating band was barely discernable. These results suggest that there is considerable homology amongst the different serotypes of *P. haemolytica* and *P. trehalosi* even in the non-conserved' amino acid regions in TbpA.

In order to evaluate the variation in the *tbpB* genes, a first attempt was made to amplify the intact genes from the various strains using specific primers for the 5 and 3 ends of the gene (primer# 401 and #199, Table 10). These primers readily amplified the intact *tbpB* gene from all the strains tested (Table 11). Restriction enzyme digestion analysis revealed that an identical digestion pattern was observed for all the seven A1 strains tested (see h44 and h196 in Figure 28, Panel B) and some strains from the other groups (h105, h104 and h98). Only subtle differences in the pattern were detected in several of the other strains (h103,h107, h99, h106, Figure 28, Panel B). Thus PCR-based approaches were used with oligonucleotide primers to variable regions to see if the variable domains found in other studies are subject to variation in *P. haemolytica*. A reverse oligonucleotide primer (#397, Table 10) was tested outside the reported conserved regions in *tbpB* (26) in combination with the 5' primer (#401, Table 10). Similarly, a forward primer (#400) from another variable region in combination with the 3 terminal oligonucleotide primer (#199) was used. The anticipated *tbpB* partial products were obtained for all the A and T strains tested except one T strain, h99 (Fig 29B and Table 11) indicating that a considerable homology does occur not only at the 5' and 3' termini of the gene but also in a number of regions known to be variable in other species.

Table 10: Oligonucleotide primers.

#	Description	Direction	Sequence
255	*tbpA* - 5' end, NdeI site at start codon	forward	CCCTATCATATGATAATGAAATATCATC
256	*tbpA* - 3' end, HindIII site after stop	reverse	TAGCGCAAGCTTCTAAAACTTCATTTCAAAT

450	*tbpA* - variable region	reverse	TAATGTTGGGCAAGTATCTTCCAC
401	*tbpB* - 5' end	forward	TAAATTAAAGGAGACATTATGTTTAAACT
199	*tbpB* - 3' end, flanking HindIII site	reverse	GCGCAAGCTTTTATTTTTCTATTTGACG
397	*tbpB* - variable region, near 3'end	reverse	CTGTTGGCAAATCTGCCAGAG
400	*tbpB* - variable region, near middle	forward	AGGTAATCGCTTTTCTGGTAAAGC

* Direction relative to orientation of coding strand for the relevant gene

Table 11. PCR-amplification of *tbpA* and *tbpB* gene segments from different serotypes of *Pasteurella haemolytica*.

Strain	Amplification of *tbpA* gene segments by oligo pair:		Amplification of *tbpB* gene segments by oligo pair:		
	Primer pair/product amplified		Primer pair/product amplified		
Strain	255/256	255/449	401/399	401/199	400/199
h44	+	+	+	+	+
h93	+	+	+	+	+
h94	+	+	+	+	+
h95	+	+	+	+	+
h96	+	+	+	+	+
h97	+	+	+	+	+
h196	+	+	+	+	+
h99	+	+	-	+	-
h100	+	+/-	+	+	+
h103	+	+	+	+	+
h104	+	+	+	+	+
h105	+	+	+	+	+
h106	+	+	+	+	+
h107	+	+	+	+	+
h174	+	+	+	+	+

Key:

+ = Product of anticipated size, comparable to the control (h196) obtained

+/- = Product of anticipated size but much weaker in intensity than the control

- = No product obtained.

Discussion

A collection of *P. haemolytica* and *P. trehalosi* isolates from other ruminants were found to be capable of acquiring iron from bovine, ovine and caprine transferrins (data not shown) which is presumed to be mediated by surface receptors that specifically bind ruminant transferrins (Al-Sultan and Aitken 1984). The collection of *P. haemolytica* (biotype A) and *P. trehalosi* (biotype T) strains involved in this study includes different serotypes (Table 9), suggesting that the receptor-mediated iron acquistion mechanism is fairly widespread within these species. There was some variability in the expression of the transferrin binding activity amongst the various strains tested under the standard conditions used for the binding assay. Similar variation in the ability to bind the conjugated host transferrin (HRP-hTf) has been observed amongst *H. influenzae* strains (Robki et al.1993). This may be partly attributable to the varying growth characteristics of the different strains. However, the highly sensitive and specific nature of the binding assay enabled definitive identification of the presence of receptor activity in the strains tested.

The competitive binding experiments and the affinity isolation experiments indicate that interaction with all three ruminant transferrins is mediated by the same receptor proteins which show considerable similarity amongst the various strains analyzed. This conclusion is further substantiated by the immunological analysis of receptor proteins prepared from a variety of *P. haemolytica* strains with monospecific sera against TbpA and TbpB (Figure 26, Panel B). Since the immunological analysis included a variety of different serotypes within biotype A and T, it would appear that the transferrin receptor proteins are fairly conserved among *P. haemolytica* disease isolates from ruminants. Although there is considerable immunological cross-rectivity amongst the two receptor proteins in a variety of different *P. haemolytica* strains, this cross-reactivity should include epitopes present at the surface *in vivo*. Preliminary results with the whole cell/antibody analysis (Figure 27) suggest the presence of such surface epitopes. The consistent pattern observed for whole cell/HRP-btf and whole cell/anti-Tbp reactivities for any given strain (Figure 27) coupled with the greatly reduced reactivity observed for both reactions in the iron replete cells suggest that the reactivity observed is against iron regulated proteins.

An important consideration in the development of an effective vaccine against any bacterial species, is the spectrum of the vaccine against the different serotypes/biotypes of the infecting bacterial species. In *N. meningitidis* while TbpA proteins are relatively homogenous, the identification of two families in the species based upon the differences in the

molecular mases and antigenic properties of their TbpB proteins (18) dictates the formulation of a heterogenous Tbp vaccine representative of the two families.

The 100Kda transferrin receptor protein (TbpA), from bovine strain of *P.haemolytica*, serotype A1 (26) was identified by the affinity purification procedure of Schryver and Morris (32). Modification of that procedure, as described in the method section, has enabled us to identify the 60Kda as the second transferrin binding protein in *P.haemolytica* analogous to TbpB in the other bacterial species (32 and Robki et al.1993). The affinity purification procedure isolated TbpA and TbpB of similar molecular weights from all of the strains of *P. haemolytica* and *P. trehalosi* examined (Figure 26, Panel A). Our results in *P. haemolytica* and *P. trehalosi* showed that antisera raised against these two purified receptor proteins from a serotype A1, strain h44, specifically recognised the receptor proteins from other A1 strains (h196, Figure 26, Panel B) and other A serotypes. These included an A11 serotype (h107) known to differ from the other A types in its reactivity with convalescent sera against the 35Kda and 70Kda iron regulated outer membrane proteins. The amounts of purified receptors from the T strains, h99 and h100 (Figure 26, Panel A) and their with the antisera (Figure 26, Panel B) appeared to be reduced compared with most of the A strains. This reduction in the antibody reactivity with the T strains was also observed in whole cell assay (Figure 27). The reduction in the whole cell-antibody reactivity in these strains, however, was consistent with their reduced reactivity with HRP-bTf (Figure 27). This suggests that the observed lower reactivity with the anti-TbpA and anti-TbpB antisera is likely due to differences in the expression of receptor proteins under the standard growth conditions used in these experiments.

The genes encoding TbpA and TbpB proteins, *tbpA* and *tbpB* are fairly homogeneous within serotype A1 (causing pneumonic pasteurellosis in cattle) and to a large extent, amongst the A types (causing pneumonia in sheep and goats) in general (Figure 28). By extending the restriction enzyme digestion analysis to the *P. trehalosi* strains, we found only marginal differences (Figure 28). The results of PCR- amplification experiments with specific 5' and 3' oligonucleotide primers and primers derived from the hypervariable regions (Figure 29, Table 11) further supports the conclusion that the *tbp* genes within *P. haemolytica* species and *P. trehalosi* are relatively homogenous. These results contrast with the differences which have been observed for *H. influenzae* and *N. meningitidis* (1 and Loosemore et al.) but will need to be confirmed by sequence analysis of genes isolated from representative strains from a number of serotypes.

The lack of evident genetic hetereogeneity in the *tbp* genes and the apparent immunological crossreactivity of the Tbp proteins from a variety of *P. haemolytica* and *P. trehalosi* strains underscore their potential as broad-spectrum vaccine antigens for prevention of infection in ruminants.

While the present invention has been described with reference to what are presently considered to be the preferred examples, it is to be understood that the invention is not

limited to the disclosed examples. To the contrary, the invention is intended to cover various modifications and equivalent arrangements included within the spirit and scope of the appended claims.

All publications, patents and patent applications are herein incorporated by reference in their entirety to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated by reference in its entirety.

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Sub H15

DETAILED FIGURE LEGENDS

~~Figure 1. Result of PCR analysis~~

a) Schematic diagram of the PCR procedure - Each circle represents a recombinant pBR322 plasmid and a possible PCR reaction. The Tbp1 primer, primer left and primer right are represented by the letters t, l and r respectively. The *Eco*RI site on the pBR322 plasmid is denoted by the letter, E. In the upper plasmid, the Tbp1 primer and primer left would amplify a PCR product corresponding to the heavy line. Similarly in the lower plasmid, the PCR product amplified by Tbp1 primer and primer right is represented by the heavy line.

(b) The 0.8 kb PCR product amplified by Tbp1 primer and primer left.

Figure 2. Restriction endonuclease map of *tbp* plasmids 9, 10 and 482. Open box - pBR322 presented linearly. Crosshatched box - PCRII presented linearly. The positions and the orientations of *tbpA*, *tbpB* are as shown by the dark arrows.

Sub H16

~~Figure 3. Preliminary nucleotide sequence of *P. haemolytica tbpA* and *tbpB*. Putative signal sequence cleavage sites are indicated by an arrow. The start codon (ATG) is underlined.~~

Figure 4. The promoter region of *P. haemolytica tbpB* (PHTBPB). The putative Fur consensus sequence is indicated by asterisks. The Fur consensus sequences of *N. gonorrhoeae tbpB* (NGTBPB) and *N. meningitidis tbpB* (NMTBPB) are also indicated.

Figure 5. Southern hybridization of *P. haemolytica* genomic DNA digested with *Cla*I and probed with the *tbpA* gene. Lanes 1-16 represent *P. haemolytica* serotypes 1 to 16. Lane M represents lambda DNA digested with *Hind* III and hybridized with lambda DNA radiolablled separately as size markers.

Figure 6. Southern hybridization of *P. haemolytica* genomic DNA digested with *Hind*III and *Bam*HI and probed with the *tbp*A gene. Lanes 1-16 represent *P. haemolytica* serotypes 1-16. The molecular sizes are as indicated on the left.

Figure 7. Southern hybridization of *A. suis* 3714, *A. pleuropneumoniae* CM5 and shope 4074 genomic DNA digested with variou restriction endonucleases and probed with *P. haemolytica tbp*A. Lane M represents lambda DNA digested with *Hind* III and hybridized with lambda DNA radiolabelled separately as size markers.

Figure 8. Restriction maps of the *tbpA*, *tbpB* regions in *P. haemolytica* A1, *A. pleuropneumoniae* CM5, Shope 4074 and *A. suis* 3714. Line 1 represents the *tbpA* probe used in Figure 15. Line 2 represents the *tbpA* probe used in Figures 16 and 17.

Sub H17

~~Figure 9. Alignment of the amino acid of Tbp1 of *P. haemolytica* A1 (PHTBP) and the Tbp1 of *N. gonorrhoeae* (NGTBP1) and *N. meningitidis* (NM1). The numbers to the right indicate amino acid positions. Asterisks indicate positions of complete identity in alignment, dots indicate similar amino acid residues. Gaps were introduced to maximize sequence alignment and are indicated by dashes (-).~~

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Sub H18

~~Figure 10. Alignment between *P. haemolytica* A1 Tbp1 (PHTBP1) and the *A. pleuropneumoniae* serotype 1 and 7 TfbA proteins (APL, APL7). Asterisks indicate positions of complete identity in alignment, dots indicatesimilar amino acid residues. Gaps were introduced to maximize sequence alignment and are indicated by dashes (-).~~

Figure 11. Dendrogram illustrating the genetic relatedness among *P. haemolytica* Tbp1 (PHTBP), *N. gonorrhoeae* Tbp1 (NGTBP1), *N. meningitidis* Tbp1 (NM1) and the TfbA proteins from *A. pleuropneumoniae* serotype 1 and 7 (APL1, APL7).

Figure 12. Peptide alignment between *P. haemolytica* A1 Tbp1 and TonB-dependent outer membrane receptors of *E. coli*. Asterisks show amino acids with complete identity in alignment, dots indicate similar amino acid residues. Gaps were introduced to maximize sequence alignment and are inidcated by dashes (-).

Figure 13. T7 analysis of the *P. haemolytica* Tbp1 protein. The molecular weight marks in kDa are as indicated on the left. Lane 1 - positive control recombinant plasmid. Lane 2 - the recombinant plasmid containing *tbpA*. Lane 3 - the vector plasmid pBluescript (SK)

Figure 14. Western immunoblot of inner and outer membranes from *P. haemolytica* A1 and *E. coli* HB101. The first antibody was a rabbit antiserum raised to the soluble antigens of *P. haemolytica* A1 and the second antibody was goat anti-rabbit alkaline phosphatase conjugate. Lane M represents the molecular weight markers in kDa. Lanes 1-4 represent outer membrane fractions and lanes 5-8 are inner membrane fractions. 6μg of protein was added to each lane. Lanes 1 and 5 - *E. coli* proteins from cells grown in LT. Lanes 2 and 6 - proteins from cells grown in BHIB. Lanes 3 and 7 - proteins from cells grown in BHIB plus 100 μM EDDA. Lanes 4 and 8 - proteins from cells grown in BHIB plus 100 μM EDDA with 1 mM $FeSo_4$ added.

Figure 15. Western immunoblot of inner and outer membranes from *P. haemolytica* A1 and *E. coli* HB101 using sera raised in calves to soluble antigens by vaccination with Presponse®. The second antibody was goat anti-bovine alkaline phosphatase conjugate. Lane M represents the molecular weight markers in kDa. Lanes 1-4 are outer membrane fractions and lanes 5-8 are inner membrane fractions. 6 μg of protein was added to each lane. Lanes 1 and 5 - *E. coli* proteins from cells grown in LT. Lanes 2 and 6 - proteins from cells grown in BHIB. Lanes 3 and 7 - proteins from cells grown in BHIB plus 100 μM EDDA. Lanes 4 and 8 - proteins from cells grown in BHIB plus 100 μM EDDA with 1 mM $FeSo_4$ added.

Figure 16. Binding of labelled transferrins by iron-deficient bacterial membranes. Aliquots of total membranes (4 μg protein) prepared from iron-deficient cells from the indicated bacterial strains were spotted onto strips of nitrocellulose/cellulose acetate paper and, after blocking, the papers wereexposed to mixtures containing 450 ng/ml of the indicated HRP-conjugated transferrin. The filters were subsequently washed and developed with HRP substrate mixture as described in the Methods section. h173, h174, h175, h176 and h44 are representative strains of *P. haemolytica* whose serotype and source are listed in Table 1. h50 - *A. equuli*. HRP-bTf,-oTf,-cTf and -eTf - HRP conjugates of bovine, ovine, caprine and equine transferrins.

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Sub H19

Figure 17. Isolation of receptor proteins with transferrin affinity columns. Affinity isolation experiments were performed with iron-deficient total membranes prepared from *P. haemolytica* strain h44 (top panel), h173 (middle panel) and h175 (bottom panel). Experiments were performed with bovine transferrin-Sepharose (lanes A and B), ovine transferrin-Sepharose (lane C), caprine transferrin-Sepharose (lane D) or equine transferrin-Sepharose (lane E) using standard washing conditions (lanes B-E) or low salt washing conditions (lane A) as outlined in the methods section. The samples eluted with buffer containing 2M guanidine HCl were dialyzed, concentrated and aliquots analyzed by SDS-PAGE and silver staining as described in the methods section.

Sub H20

Figure 18. Immunological analysis of receptor proteins from different serotypes of *P. haemolytica* from bovine, sheep and goats. Aliquots of purified receptor proteins from representative serotypes of *P. haemolytica* were subjected to SDS-PAGE, electroblotted and then probed with specific anti-TbpB serum (Panel A) or with anti-TbpA serum (Panel B) as described in the methods section. The following *P. haemolytica* strains of the indicated serotype were included in the analysis; Lane 1 - strain h44 (A1), Lane 2 - h173 (untypable), Lane 3 - h175 (A7), Lane 4 - h176 (A9), Lane 5 - h100 (T4), Lane 6 - h106 (T10), and Lane 7 - h107 (A11). The numbers on the left represent the molecular weights (X 1000) of standard proteins.

Sub H21

Figure 19. Binding of labelled transferrin and anti-receptor antibody by intact cells. The indicated bacterial strains were grown under iron-limiting conditions, harvested by centrifugation and resuspend to a A_{600} of 1-2 in 50 mM TrisHCl, 150 mM NaCl, pH 7.5 buffer. A 5 µl aliquot of the suspensions were applied to HA membrane, the membrane was dried, blocked and then exposed to blocking solution containg labelled transferrin (HRP-bTf) or antireceptor antibody (anti-TbpA, anti-TbpB). The latter membranes were washed and subsequently exposed to labelled second antibody prior to development with substrate.

Sub H22

Figure 20. Map of the P. haemolytica tbp operon (Top) and regulatory sequences (Bottom). tbpA and tbpB are the genes encoding for TbpA and TbpB, respectively; p, is the putative promoter region preceding tbpB and denoted as -35 and -10 sites at the bottom. A putative Fur box is represented as two arrows in opposite directions in the sequence at the bottom; rnaseT and fis are two ORFs flanking the P.haemolytica tbp operon encoding for proteins highly homologous to E. coli and H. influenzae RNase transferase and factor-for-invertion stimulation proteins, respectively. Additionally, putatives ribosomal binding site or Shine-Dalgarno (SD) consensus sequence, transcriptional start (Met), and stop codons (SC) are also bolded.

Figure 21 . The DNA sequence of the *tbpA* gene from *P. haemolytica* strain H196.

Figure 22. Predicted amino acid sequence of the TbpA protein from *Pasteurella haemolytica* strain H196. Italicized amino acids correspond to the experimentaly determined N-terminal amino acids of the mature protein. Residues indicated by strikethrough constitute the leader peptide region. Residues that are identical in TbpAs from Neisseria meningitidis, N. gonorrhoeae, H. influenzae and Actinobacillus pleuropneumoniae are bold and underlined.

Regions proposed as internal segments (dark shading), intermembrane b-strands (light shading) or external segments (no shading) based on the proposed topology model by Tommassen (28) are indicated.

Figure 23. The DNA sequence ot the *tbpB* gene from *P. haemolytica* strain H196.

Figure 24. Predicted amino acid sequence of the TbpB protein from *P. haemolytica* strain H196. Italicized amino acids correspond to the predicted N-terminal amino acids of the mature protein. Residues indicated by strikethrough constitute the leader peptide region. Regions of homology are identified by shading and residues that are identical in TbpBs from Neisseria meningitidis, N. gonorrhoeae, H. influenzae and Actinobacillus pleuropneumoniae are bold and underlined.

Figure 25. Solid-phase HRP-Tf binding assay. Total membrane preparations from the indicated strains of *P. haemolytica* and *P.trehalosi* were spotted onto nitrocellulose-cellulose acetate paper and blocked with skim milk prior to incubation with HRP-bTf, -oTf or -gTf. Binding was detected with chloronaphthol reagent as in the methods.The letters on the left indicate the strains while the letters on the top indicate the different HRP-labbelled ruminant transferrins.

~~**Figure 26.** Western blot cross-reactivity studies with anti-TbpA and anti-TbpB antisera from *P.haemolytica* serotype A1. Aliquots of affinity purified receptor proteins from the indicated strains of *P.haemolytica* and *P. trehalosi* were separated by SDS-PAGE and Silver stain (panel A) or subjected to Western blotting(panel B) as in Methods. Tbp proteins were identified by incubation with a mixture of anti-TbpB(1/1000) and anti-TbpA(1/1000) rabbit antisera as described in the methods section. The numbers on the left represent the molecular weights (X 1000) of standard proteins in kilodaltons.~~

Figure 27. Cross-reactivity studies with monospecific anti-TbpA and anti-TbpB antisera from *P. haemolytica* serotype A1 against intact cells. Aliquots of iron-limited intact cells from the indicated strains of *P. haemolytica* and *P. trehalosi* were spotted onto HA nitrocellulse paper and, after blocking, the membrane was either exposed to HRP - labelled bTf, anti-TbpA antiserum or anti-TbpB antiserum . Bound antibodies were subsequently detected by labelled goat anti-rabbit antibody as described in the methods. A preparation of intact *P. haemolytica* strain h44 (type A1) cells grown under iron-sufficient cells (indicated by h44 - Fe^+) was spotted onto the membranes and used as a control.

~~**Figure 28.** Restriction endonuclease digestion pattern of PCR-amplified *tbpA* (Panel A) and TbpB (Panel B) genes from *P. haemolytica* and *P.* trehalosi strains. The *tbp* genes amplified by colony PCR from the indicated strains were digested with Sau3A1 restriction endonuclease. The resulting digests were electrophoresed on a 7.5% polyacrylamide gel as described in Methods. The letters above the lanes indicate the source strain template DNA used in PCR while the letters on the left indicate the molecular weight standard in kilobases. Imaging was done with a Hewlett-Parkard ScanJet IIp. In fig 29B, primer #s 397 and 400 from non-conserved regions of~~

P.hamolytica tbpB gene, were used in combination with opposing primers {#s 401(5′) and 199(3′)} respectively.

Figure 29. PCR amplification of variable segments of the *tbpA* and *tbpB* genes. For *tbpA* gene , oligonucleotide primer # 450 made from the deduced amino acid sequence from a hypervariable region of tbpA was used in combination with the 5′ specific primer (#255) to amplify the gene segment from the various P.haemolytica strains . The products were then analysed on 1% agarose gel followed by staining with ethidium bromide.

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